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MECHANISM-BASED INHIBITION OF CYP2C8 BY GEMFIBROZIL IN HUMANS: CHARACTERISATION OF TIME AND DOSE RELATIONSHIPS

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ACADEMIC DISSERTATION

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ABBREVIATIONS AND DEFINITIONS

ATP	Adenosine triphosphate
AUC	Area under concentration-time curve
$AUC_{0-\infty}$	Area under concentration-time curve from time 0 to infinity
AUC_{0-t}	Area under concentration-time curve from time 0 to t
AUC_c	Area under concentration-time curve in control phase
AUC_i	Area under concentration-time curve in inhibited phase
CAR	Constitutive androstane receptor
$C_{h,u}/C_{p,tot}$	Ratio of unbound hepatic to plasma total concentration
CI	Confidence interval
CL	Clearance
CL/F	Oral clearance
$C_{avg,t}$	Average concentration during time 0-t
C_{max}	Maximum concentration
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
F	Oral bioavailability
FDA	Food and Drug Administration
FDCL	Fractional decrement in oral clearance
F_G	Extent of bioavailability across the intestinal wall
f_m	Fraction metabolised by an enzyme
$f_{m,CYP2C8}$	Fraction metabolised by CYP2C8
$f_{t,OATP1B1}$	Fraction transported by OATP1B1
f_u	Fraction unbound
GR	Glucocorticoid receptor
HLM	Human liver microsomes
HMG CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HUCH	Helsinki University Central Hospital
[I]	Inhibitor concentration
IC_{50}	Inhibitor concentration supporting half of the maximal inhibition
$[I]_g$	Inhibitor concentration in the intestine
$[I]_h$	Inhibitor concentration in the liver
$[I]_{p,u}$	Plasma unbound inhibitor concentration
IVIVE	*In vitro – in vivo* extrapolation
k_{deg}	First-order degradation rate constant of an enzyme
$k_{deg,gut}$	First-order degradation rate constant of an enzyme in the intestine
$k_{deg,hep}$	First-order degradation rate constant of an enzyme in the liver
k_e	Elimination rate constant
K_i	Inhibitor concentration supporting half of the maximal inhibition
k_{inact}	Maximal rate of inactivation
K_I	Inhibitor concentration supporting half of the maximal rate of enzyme inactivation

K_m	Michaelis-Menten kinetic constant
k_{obs}	Apparent rate of inactivation
MBI	Mechanism-based inhibition
MRP2	Multidrug resistance-associated protein 2
MDMA	3,4-dimethylenedioxymetamphetamine (ecstasy)
MIC	Metabolic inhibitory complex / metabolic intermediate complex
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NTCP	Sodium-dependent taurocholate cotransporting polypeptide
OAT	Organic anion transporter
OATP	Organic anion-transporting polypeptide
OCT	Organic cation transporter
P-gp	P-glycoprotein
PBPK	Physiologically based pharmacokinetic
Ph. Eur.	European Pharmacopoeia
PPARα	Peroxisome proliferator activated receptor alpha
PXR	Pregnane X receptor
r	Partition ratio
r^2	Coefficient of determination
[S]	Substrate concentration
SD	Standard deviation
SLCO	Solute carrier organic anion transporter
SNP	Single nucleotide polymorphism
t	Time
$t_{1/2}$	Elimination half-life
TDI	Time-dependent inhibition
t_{max}	Time to maximum concentration
UGT	Uridine-5’-diphosphoglucuronosyltransferase
v	Rate of metabolite formation
V_d	Volume of distribution
V_{max}	Maximum velocity

LIST OF ORIGINAL PUBLICATIONS

This thesis work is based on the following original publications, which are referred to in the text by the Roman numerals I-IV.

I Johanna Honkalammi, Mikko Niemi, Pertti J. Neuvonen and Janne T. Backman. Mechanism-based inactivation of CYP2C8 by gemfibrozil occurs rapidly in humans. *Clin Pharmacol Ther* 89: 579-86 (2011).

II Janne T. Backman, Johanna Honkalammi, Mikko Neuvonen, Kaisa J. Kurkinen, Aleksi Tornio, Mikko Niemi and Pertti J. Neuvonen. CYP2C8 activity recovers within 96 hours after gemfibrozil dosing: Estimation of CYP2C8 half-life using repaglinide as an *in vivo* probe. *Drug Metab Dispos* 37: 2359-66 (2009).

III Johanna Honkalammi, Mikko Niemi, Pertti J. Neuvonen and Janne T. Backman. Dose-dependent interaction between gemfibrozil and repaglinide in humans: Strong inhibition of CYP2C8 with subtherapeutic gemfibrozil doses. *Drug Metab Dispos* 39: 1977-86 (2011).

IV Johanna Honkalammi, Mikko Niemi, Pertti J. Neuvonen and Janne T. Backman. Gemfibrozil is a strong inactivator of CYP2C8 in very small multiple doses. *Clin Pharmacol Ther* (in press).

ABSTRACT

Drug-drug interactions may cause serious, even fatal clinical consequences. Therefore, it is important to examine the interaction potential of new chemical entities early in drug development. Mechanism-based inhibition is a pharmacokinetic interaction type, which causes irreversible loss of enzyme activity and can therefore lead to unusually profound and long-lasting consequences. The *in vitro – in vivo* extrapolation (IVIVE) of drug-drug interactions caused by mechanism-based inhibition is challenging. Consequently, many of these interactions have remained unrecognised for many years.

The concomitant use of the fibrate-class lipid-lowering agent gemfibrozil increases the concentrations of some drugs and their effects markedly. Even fatal cases of rhabdomyolysis occurred in patients administering gemfibrozil and cerivastatin concomitantly. One of the main mechanisms behind this effect is the mechanism-based inhibition of the cytochrome P450 (CYP) 2C8 enzyme by a glucuronide metabolite of gemfibrozil leading to increased cerivastatin concentrations. Although the clinical use of gemfibrozil has clearly decreased during recent years, gemfibrozil is still needed in some special cases. To enable safe use of gemfibrozil concomitantly with other drugs, information concerning the time and dose relationships of CYP2C8 inhibition by gemfibrozil should be known.

This work was carried out as four *in vivo* clinical drug-drug interaction studies to examine the time and dose relationships of the mechanism-based inhibitory effect of gemfibrozil on CYP2C8. The oral antidiabetic drug repaglinide was used as a probe drug for measuring CYP2C8 activity in healthy volunteers. In this work, mechanism-based inhibition of the CYP2C8 enzyme by gemfibrozil was found to occur rapidly in humans. The inhibitory effect developed to its maximum already when repaglinide was given 1-3 h after gemfibrozil intake. In addition, the inhibition was shown to abate slowly. A full recovery of CYP2C8 activity, as measured by repaglinide metabolism, was achieved 96 h after cessation of gemfibrozil treatment. The dose-dependency of the mechanism-based inhibition of CYP2C8 by gemfibrozil was shown for the first time in this work. CYP2C8 activity was halved by a single 30 mg dose of gemfibrozil or by twice daily administration of less than 30 mg of gemfibrozil. Furthermore, CYP2C8 activity was decreased over 90% by a single dose of 900 mg gemfibrozil or twice daily dosing of approximately 100 mg gemfibrozil. In addition, with the application of physiological models to the data obtained in the dose-dependency studies, the major role of mechanism-based inhibition of CYP2C8 in the interaction between gemfibrozil and repaglinide was confirmed.

The results of this work enhance the proper use of gemfibrozil and the safety of patients. The information related to time-dependency of CYP2C8 inhibition by gemfibrozil may also give new insights in order to improve the IVIVE of the drug-drug interactions of new chemical entities. The information obtained by this work may be utilised also in the design of clinical drug-drug interaction studies in the future.

INTRODUCTION

Drug-drug interactions may have serious clinical consequences, and therefore, the potential of new chemical entities causing or being a victim of an interaction should be carefully studied. The interaction potential can be assessed using *in vitro* (laboratory), *in vivo* (animal and human study) and *in silico* (computational) methods. In early drug development, *in vitro* methods are used for assessing the metabolic pathways and for screening the interaction potential (Pelkonen *et al*., 2005). *In vitro - in vivo* extrapolation (IVIVE) is used for predicting the clinical drug-drug interactions of the compound. In case of signs of interaction potential based on *in vitro* studies or IVIVE, drug interaction studies are carried out.

Two decades ago, gemfibrozil, a fibric acid derivative, was a promising agent for the treatment of hyperlipidaemia. It was shown to reduce triglyceride levels and increase high-density lipoprotein cholesterol levels, and to reduce the number of coronary events in patients with or without a history of coronary disease. However, no reduction in the total mortality in gemfibrozil-treated subjects could be seen (Frick *et al*., 1987). Gemfibrozil seemed safe and well-tolerated, but turned out to cause serious drug-drug interactions. E.g., patients administering concomitantly cerivastatin and gemfibrozil developed myopathy more often than when using the drugs separately (Furberg and Pitt, 2001). Approximately one third of the cerivastatin treated patients who developed fatal rhabdomyolysis had used gemfibrozil concomitantly.

The interaction potential of gemfibrozil is complex. Gemfibrozil has been shown to increase the exposure to several drugs, e.g., repaglinide, cerivastatin, rosiglitazone, pioglitazone, montelukast and loperamide in humans (Backman *et al*., 2002; Niemi *et al*., 2003a; Niemi *et al*., 2003b; Jaakkola *et al*., 2005; Niemi *et al*., 2006; Karonen *et al*., 2010). According to the current understanding, gemfibrozil can inhibit both drug metabolism and transport, thereby affecting the elimination of drugs from the body. Although the complex interaction potential of gemfibrozil has restricted its clinical use, it still has a role in the management of hyperlipidaemia in certain cases (Loomba and Arora, 2010).

The interaction potential of gemfibrozil is utilised in the examination of the metabolic profile and interaction potential of new drug candidates. Gemfibrozil is recommended as CYP2C8 inhibitor both in *in vitro* and *in vivo* studies in drug development by regulatory authorities (FDA, 2006; EMA, 2010). The inhibition of the CYP2C8 enzyme by gemfibrozil is mainly based on mechanism-based inactivation of CYP2C8 by the glucuronide metabolite of gemfibrozil (Shitara *et al*., 2004; Ogilvie *et al*., 2006). Both the inhibition mechanism and the fact that it is caused by a phase II metabolite of a drug make this interaction unique (VandenBrink and Isoherranen, 2010).

The mechanism-based nature of CYP2C8 inhibition by gemfibrozil has been earlier reported and the inhibition mechanism has been kinetically characterised in *in vitro* studies (Ogilvie *et al*., 2006). In addition, the effect of gemfibrozil on many CYP2C8 substrate drugs has been studied *in vivo* in humans (Backman *et al*., 2002; Niemi *et al*., 2003a; Niemi *et al*., 2003b;

Jaakkola *et al*., 2005; Niemi *et al*., 2006; Ogilvie *et al*., 2006). In a recent study, the effect of gemfibrozil on CYP2C8-mediated metabolism in humans persisted at least for 12 h after discontinuation of gemfibrozil (Tornio *et al*., 2008a). It created us to examine the persistence of the CYP2C8 inhibition by gemfibrozil in more detail. It could be hypothesised also that the interaction would initiate fairly slowly. Firstly, gemfibrozil is a mechanism-based inhibitor. Secondly, the interaction between gemfibrozil and CYP2C8 substrates is mainly based on a phase II metabolite of an orally administered drug. To examine this issue, we studied the onset time of mechanism-based CYP2C8 inhibition by gemfibrozil in humans.

In addition to time-dependency, the dose-dependency of the mechanism-based inhibition of CYP2C8 by gemfibrozil has not been known. Only a few studies concerning dose-dependency of mechanism-based inhibitors in humans have been published, and none of them concerned gemfibrozil. In this thesis work, based on studies using gemfibrozil at different dose levels new data concerning the interaction mechanism was obtained. The time- and dose-dependency information of mechanism-based inhibition obtained in this work may guide the clinical use of gemfibrozil and CYP2C8 substrate drugs, and aid the development process of new therapeutic drugs in the future.

REVIEW OF THE LITERATURE

1. Drug metabolism and pharmacokinetic interactions

Drugs are foreign compounds to the human body. Most of them are relatively hydrophobic and need to undergo biotransformation in order to terminate their therapeutic effect and facilitate their elimination from the body (Murray, 1997). Biotransformation of drugs, which protects the human body from intoxications (Remmer, 1965; Remmer, 1970), can be divided into two major groups of reactions (Meyer, 1996). Phase I reactions, e.g., oxidation, reduction, hydrolysis and hydration, introduce or expose a functional group in the drug molecule. This functional group is subsequently conjugated, often with a highly water soluble moiety such as glucuronic or sulfonic acid, during phase II metabolism. In addition to the protective detoxification function, biotransformation may also cause formation of active intermediate species, which, in certain situations, may elicit tissue lesions (Remmer, 1970; Brodie *et al*., 1971; Murray, 1997).

1.1. Cytochrome P450 enzymes

Cytochrome P450 (CYP) enzymes are the most important phase I enzymes. They are a superfamily of haem containing polypeptide chains, which catalyse primarily oxidative metabolism of drugs and other xenobiotics (Wrighton and Stevens, 1992; Brown *et al*., 2008). In addition to drug and other xenobiotic biotransformation, CYP enzymes have key roles in physiological processes, e.g., bile acid biosynthesis, cholesterol metabolism as well as steroid and vitamin D biosynthesis and metabolism (Nebert and Russell, 2002; Estabrook, 2003). CYP enzymes are mainly located in the liver and in the gut, which are the main organs for drug metabolism, but they are found in other tissues as well (Krishna and Klotz, 1994).

Cytochrome P450 was detected in the 1950's and first thought to be one single enzyme, which was associated with the metabolism of drugs and steroids (Nebert and Russell, 2002). Later, it was understood that several different CYP enzymes exist. As the number of different CYP enzymes increased, a nomenclature system was considered necessary. The nomenclature system introduced in 1987 arranges CYP enzymes into families and subfamilies based on the amino acid sequences (Nelson *et al*., 1996). Enzymes that share ≥ 40% identity belong to a family designated by an Arabic numeral (e.g., CYP2) and enzymes that share ≥ 55% identity comprise a subfamily designated by a letter (e.g., CYP2C). Individual enzymes are separated from other members in the same subfamily with a unique Arabic number at the end (e.g., CYP2C8). The CYP families participating in the metabolism of drugs and other xenobiotics are almost exclusively CYP1, CYP2 and CYP3 (Nebert and Russell, 2002). CYP2C8 is presented in more detail in section 8.

The human CYP superfamily consists of altogether 57 genes. The genes of the CYP enzymes are written in italics, and the reference allele with efficient metabolic activity is named with **1* (e.g., *CYP2C8*1*) (Nebert and Russell, 2002). Allelic variants, which may have different metabolic activities, are named with different numbers and/or letters, e.g., **2* or **1B*. By

definition, allelic variants are considered genetic polymorphisms, if they occur in ≥ 1% of the population (Nelson, 1999).

In addition to structure, CYP enzymes differ from each other, e.g., by expression site and quantity, substrate specificity and susceptibility to different enzyme inhibitors and inducers (Guengerich, 1992). CYP enzymes are located in the endoplasmic reticulum of the cells (Meyer, 1996). The basic catalytic cycle of CYP enzymes is shown in Figure 1 (Hollenberg, 1992; Lin and Lu, 1998).

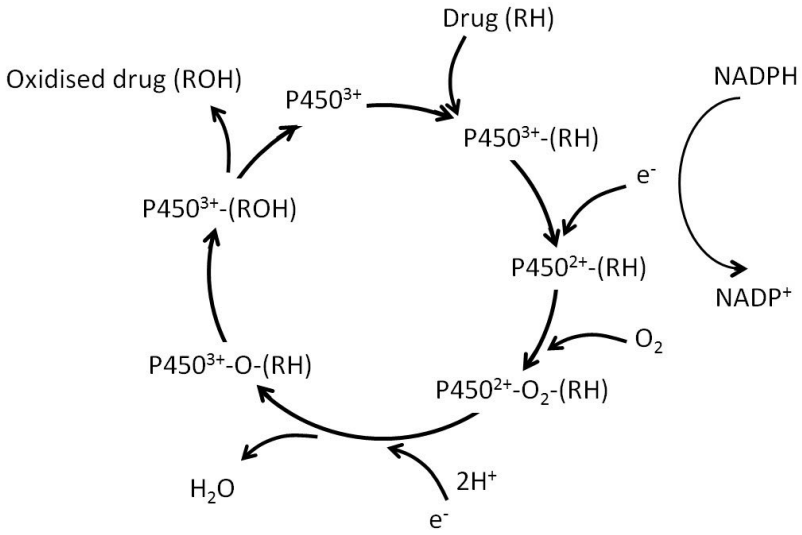

Figure 1. Basic catalytic cycle of cytochrome P450 enzymes. RH, drug; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, nicotinamide adenine dinucleotide phosphate; e^-, electron; ROH, oxidised drug.

1.2. Pharmacokinetic drug interactions

Main mechanisms of pharmacokinetic interactions include physicochemical interactions, plasma protein binding related interactions, as well as interactions due to changes in enzyme and transporter activity (Kanamitsu *et al*., 2000b). Both increase and decrease in enzyme and transporter activities can occur, due to induction and inhibition, respectively. Pharmacokinetic interaction mechanisms can lead to changes in the absorption, distribution, metabolism and excretion of the drugs. Inhibition of CYP enzymes is one of the main mechanisms leading to clinical drug-drug interactions (Lin and Lu, 1998; Pelkonen *et al*., 2008).

2. Enzyme inhibition

Drug metabolism plays an important role *in vivo*, and its inhibition may cause severe consequences. The inhibition of drug metabolism can lead to decreased clearance (CL), and therefore, increased exposure to the parent compound. This may lead to enhanced drug efficacy and even fatal toxicity or, in the case of prodrugs, to decreased therapeutic effect (Lin and Lu, 1998; Pelkonen *et al*., 1998; Pelkonen *et al*., 2008). The consequences of enzyme inhibition *in vivo* may vary due to many reasons, e.g., the inhibitor drug (e.g., inhibition type,

inhibitor potency, dosing scheme), the victim drug (e.g., the importance of the inhibited enzyme for metabolism, presence of alternative metabolic routes, therapeutic index) and the patient (e.g., concomitant diseases, concomitant medications, variability in drug metabolism).

The area under the concentration-time curve (AUC) reflects the *in vivo* exposure to the drug. The fold increase in the AUC of the parent drug in the inhibited state compared to control phase ($AUC_{inhibited}/AUC_{control}$) can be used as a simple method for the quantification of the enzyme inhibition *in vivo* (Ito *et al*., 2004). With few exceptions, changes in exposure of less than 2-fold typically occur without clinical consequences, whilst an increase of the exposure to a drug by 2-fold or more is considered a threshold for greater concern (Obach, 2009). However, the therapeutic index of the victim drug affects the clinical relevance of the enzyme inhibition markedly, and even a small change in exposure may be relevant with drugs having a narrow therapeutic range.

Based on the reversibility of the inhibitory effect, CYP enzyme inhibition can be divided into reversible, quasi-irreversible and irreversible inhibition (Hollenberg, 2002).

2.1. Reversible inhibition

Reversible inhibition arises from the competition of the inhibitor and the substrate of the metabolising enzyme. Reversible enzyme inhibition is believed to involve only the first step of the CYP catalytic cycle (binding to the ferric form of the enzyme). The inhibitor is non-covalently bound to the enzyme, and can dissociate from it making the inhibition reversible. Reversible inhibition actually disappears soon after the removal of the inhibitory agent, e.g., due to clearance of the drug from the body or due to filtration, centrifugation or electrophoresis in experimental conditions. Both the onset and offset of reversible inhibition are rapid, governed mainly by the inhibitor concentration at the enzyme site. The potency of the inhibitor can be described with the inhibitor concentration needed for half of the maximal inhibition (K_i).

The velocity of the CYP mediated enzymatic reactions *in vitro* can usually be described using the classic Michaelis-Menten equation:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where v is the rate of metabolite formation, V_{max} is the maximum velocity, K_m is the Michaelis-Menten constant describing the substrate concentration at half-maximal velocity, and [S] is the substrate concentration.

Known metabolic index reactions can be used in the assessment of the activity of different CYPs *in vitro*. A decrease in the formation of the expected metabolites, or a decrease in the depletion of the parent compound in the index reactions in the presence of the compound under examination serves as an indicator of enzyme inhibition (Pelkonen and Turpeinen, 2007; Emoto *et al*., 2010). Some index reactions for the main human drug metabolising CYPs are presented in Table 1.

Table 1. Index reactions for CYP enzyme activity (Guengerich, 1990; Bjornsson *et al*., 2003; Testino and Patonay, 2003; Walsky and Obach, 2004; Pelkonen *et al*., 2005; Turpeinen *et al*., 2005b).

CYP enzyme	*Index reaction*	*CYP enzyme*	*Index reaction*
CYP1A2	Caffeine N-demethylation Melatonin 6-hydroxylation Phenacetin O-deethylation	CYP2C19	(S)-mephenytoin 4'-hydroxylation Omeprazole 5'-hydroxylation
CYP2A6	Coumarin 7-hydroxylation	CYP2D6	Bufuralol 1'-hydroxylation Dextromethorphan O-demethylation
CYP2B6	Bupropion hydroxylation	CYP2E1	Chlorzoxazone 6-hydroxylation N,N-dimethylnitrosoamine N-demethylation
CYP2C8	Amodiaquine N-deethylation Paclitaxel 6α-hydroxylation	CYP3A4	Midazolam 1'-hydroxylation Testosterone 6β-hydroxylation
CYP2C9	Tolbutamide 4-hydroxylation Diclofenac 4'-hydroxylation Torsemide tolyl methylhydroxylation S-warfarin 7-hydroxylation		

Reversible inhibition can be divided into subcategories of competitive, noncompetitive and uncompetitive inhibition by the inhibitory mechanism.

Competitive inhibition. In competitive inhibition, the inhibitor prevents the binding of the substrate to the active site of the enzyme, i.e., the inhibitor and the substrate compete for the same binding site. This is the most common form of reversible inhibition affecting drug metabolism. The enzyme kinetics in competitive inhibition can be described according to the equation below:

$$v = \frac{V_{max} \cdot [S]}{K_m \cdot \left(1 + \frac{[I]}{K_i}\right) + [S]}$$

where [I] is the inhibitor concentration (Segel, 1975). This type of inhibition can be overcome by increasing the concentration of the substrate.

Noncompetitive inhibition. In noncompetitive inhibition, the inhibitor and the substrate bind to different sites of the enzyme, and the inhibitor has no effect on the binding of the substrate to the enzyme. However, the complex formed by the enzyme, inhibitor and substrate is nonproductive. The enzyme kinetics in noncompetitive inhibition can be described according to the equation below (Segel, 1975):

$$v = \frac{V_{max} \cdot [S]}{K_m \cdot \left(1 + \frac{[I]}{K_i}\right) + [S] \cdot \left(1 + \frac{[I]}{K_i}\right)}$$

Increasing the substrate concentration does not decrease the effect of this inhibition type. Noncompetitive inhibition is seen less frequently than competitive inhibition in drug metabolism.

Uncompetitive inhibition. In uncompetitive inhibition, the inhibitor does not bind to the free enzyme, but binds to the substrate-bound enzyme making the enzyme-substrate-inhibitor complex unproductive. This type of inhibition is seldomly seen in drug metabolism. The enzyme kinetics in uncompetitive inhibition can be described according to the following equation (Segel, 1975):

$$v = \frac{V_{max} \cdot [S]}{K_m + [S] \cdot \left(1 + \frac{[I]}{K_i}\right)}$$

In addition to the types presented above, also mixed-type reversible inhibition can occur. The changes in the kinetic constants in different subtypes of reversible inhibition are shown in Table 2 (Fowler and Zhang, 2008).

Table 2. Changes in the kinetic constants in different types of reversible inhibition.

Inhibition type	*Change in V_{max}*	*Change in K_m*
Competitive	-	↑
Mixed competitive/noncompetitive	↓	↑/↓
Noncompetitive	↓	-
Uncompetitive	↓	↓

V_{max}, maximum velocity; K_m, Michaelis-Menten kinetic constant

2.2. Quasi-irreversible and irreversible inhibition

Quasi-irreversible and irreversible inhibitions require at least one catalytic cycle of the enzyme to produce reactive intermediate species that modify the enzyme and cause loss of enzyme function.

In irreversible inhibition, the victim enzyme catalyses the inhibitor into an active species, which, prior to its release from the active site, inactivates the enzyme. The inactivated enzyme is thereby prevented from catalysing its reaction permanently. This phenomenon usually results from the formation of a covalent bond between the inhibitor and the enzyme (Silverman, 1995).

In quasi-irreversible CYP inhibition, or metabolic inhibitory complex (MIC) formation, a covalent bond is not produced, but a pair of electrons is donated from the inhibitor leading to sequestration of the enzyme to a functionally inactive state. Quasi-irreversible inhibition can be reversed in experimental conditions, e.g., by incubation with highly lipophilic drugs that displace the metabolic intermediate from the active site of the enzyme, by irradiation at 400-500 nm, or by ferricyanide (Ullrich and Schnabel, 1973; Dickins *et al*., 1979), but is

irreversible in physiological conditions and is therefore indistinguishable from the pharmacokinetic impacts of irreversible inhibition *in vivo* (Riley *et al*., 2007).

The phenomena of quasi-irreversible and irreversible inhibitions depending on the mechanism of the inhibited enzyme are called mechanism-based inhibition (MBI). In addition to mechanism-based enzyme inhibition, the reactive intermediate species produced by the enzyme may lead to organ toxicity such as hepatotoxicity induced by tienilic acid or troglitazone (Brodie *et al*., 1971; Ortiz de Montellano and Correia, 1983; Hollenberg, 1992; Cohen *et al*., 1997; Walgren *et al*., 2005; Masubuchi and Horie, 2007).

3. Mechanism-based inhibition

3.1. History and clinical relevance

Mechanism-based inhibitors, also called "suicide inhibitors" and "Trojan horse inactivators", are substrate molecules for the target enzyme, which in the process of catalytic conversion are changed into intermediates or products that inactivate the enzyme (Rando, 1984). This phenomenon detected in the late 1960's (Helmkamp *et al*., 1968) and initially used in enzymology is particularly common in CYP-mediated biotransformations (Jones and Hall, 2002). In mechanism-based inhibition the inactivator irreversibly alters the enzyme and removes it permanently from the pool of active enzyme. Synthesis of enzyme is needed for regaining the enzymatic activity (Figure 2).

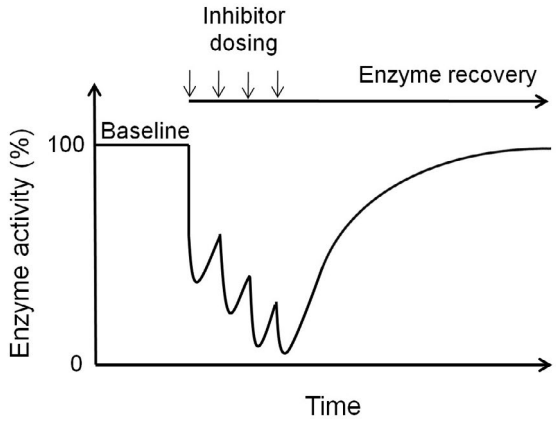

Figure 2. Changes in enzyme activity in mechanism-based inhibition *in vivo*.

As the *in vivo* enzyme activity after mechanism-based inhibition can be regained only by *de novo* synthesis of the protein, its consequences can be more profound and persist longer than those of reversible inhibition. In fact, among the drugs causing pharmacokinetic interactions, mechanism-based inhibitors represent several of those agents causing interactions of the greatest magnitude (Venkatakrishnan *et al*., 2007).

The observation made in the 1980's that the effects of the narcotic agent alfentanil were prolonged when patients had used the antibacterial agent erythromycin concomitantly was first shown to be due to the inhibition of alfentanil metabolism (Bartkowski *et al*., 1989), and later the mechanism was specified to be a mechanism-based inhibition of CYP3A4 by erythromycin (Periti *et al*., 1992). Similarly, the accumulation of caffeine in healthy volunteers treated concomitantly with antiasthmatic drug furafylline was first understood to be caused by the inhibition of caffeine metabolism by furafylline (Tarrus *et al*., 1987), and only later furafylline was shown to be a mechanism-based inhibitor of CYP1A2 (Kunze and Trager, 1993).

The first reports of fatal clinical drug-drug interactions understood to be caused by the mechanism-based inhibition of drug metabolising enzymes were from 15 Japanese cancer patients who died of toxic effects of 5-fluorouracil during concomitant administration of 5-fluorouracil prodrugs and the antiviral drug sorivudine. Sorivudine was later shown to be a mechanism-based inhibitor of dihydropyrimidine dehydrogenase, an enzyme responsible for 5-fluorouracil metabolism (Okuda *et al*., 1997).

Since then many clinically important drugs have been shown to be mechanism-based inhibitors of drug metabolising enzymes (Jones *et al*., 1999). The development of otherwise promising drugs has been stopped due to mechanism-based inhibition, and even approved drugs have been withdrawn from the market because of it. E.g., the development of furafylline was stopped early due to the mechanism-based inhibition of CYP1A2, and the calcium-channel blocker mibefradil was withdrawn from the market shortly after introduction to medical practice in 1998 due to the mechanism-based inhibition of CYP3A causing severe drug-drug interactions (Mullins *et al*., 1998; Backman *et al*., 1999; Prueksaritanont *et al*., 1999; Tucker *et al*., 2001). Many important drugs have been identified as mechanism-based inhibitors of CYP enzymes, but have been preserved in clinical use with an understanding and careful management of this property. Examples of drugs, which are or have been in clinical use, and are mechanism-based inhibitors of CYP enzymes, are provided in Table 3.

In addition, other compounds than therapeutic agents, e.g., industry chemicals, illicit drugs (e.g., 3,4-dimethylenedioxymetamphetamine (MDMA; ecstasy)), and natural compounds in food and herbal medicines (e.g., flavonoids bergamottin and 6',7'-dihydroxybergamottin in grapefruit juice and glabridin in licorice root) can inactivate enzymes by this mechanism (Halmes *et al*., 1997; Lown *et al*., 1997; Schmiedlin-Ren *et al*., 1997; Wu *et al*., 1997; Guo *et al*., 2000; de la Torre *et al*., 2004; Heydari *et al*., 2004).

Mechanism-based inhibition affecting human drug metabolism takes mainly place in the gut and the liver, which are the main organs responsible for drug metabolism, but it has also been observed to occur in other tissues, e.g., in the lungs (Murai *et al*., 2010).

Table 3. Examples of drugs with mechanism-based inhibition potential of CYP enzymes.

Drug	*Therapeutic use*	*Inactivated CYP enzyme*	*Reference(s)*
Clarithromycin	Antibacterial	CYP3A4	(Franklin, 1991; Mayhew *et al*., 2000)
Clopidogrel	Antithrombotic	CYP2B6 and CYP2C19	(Richter *et al*., 2004; Turpeinen *et al*., 2005a)
Diltiazem	Cardiovascular	CYP3A4	(Ma *et al*., 2000; Rowland Yeo and Yeo, 2001)
Disulfiram	Alcoholism	CYP2E1	(Kharasch *et al*., 1993)
Domperidone	Anti-emetic	CYP3A4	(Chang *et al*., 2010)
Erythromycin	Antibacterial	CYP3A4	(Franklin, 1991; Periti *et al*., 1992)
Ethinylestradiol	Contraceptive	CYP3A4*	(Guengerich, 1988; Lin *et al*., 2002)
Fluoxetine	Antidepressant	CYP3A4	(Mayhew *et al*., 2000)
Fluticasone	Anti-inflammatory (glucocorticoid)	CYP3A5	(Murai *et al*., 2010)
Gemfibrozil (1-O-β-glucuronide metabolite)	Anti-lipidaemic	CYP2C8	(Ogilvie *et al*., 2006)
Gestodene	Contraceptive	CYP3A4*	(Guengerich, 1990)
Imatinib	Antineoplastic	CYP3A4	(Filppula *et al*., 2011a)
Isoniazid	Antitubercular	CYP1A2, CYP2A6, CYP2C8, CYP2C19 and CYP3A4	(Wen *et al*., 2002b; Polasek *et al*., 2004)
Lapatinib	Antineoplastic	CYP3A4	(Teng *et al*., 2010)
Lopinavir	Antiviral	CYP3A4	(Ernest *et al*., 2005)
Mibefradil	Cardiovascular	CYP3A4	(Prueksaritanont *et al*., 1999)
Noscapine	Antitussive	CYP2C9 and CYP3A4	(Fang *et al*., 2010)
Omeprazole	Treatment of gastro-oesophageal acidity	CYP2C19	(Paris *et al*., 2008; Ogilvie *et al*., 2011)
Paroxetine	Antidepressant	CYP2D6	(Bertelsen *et al*., 2003)
Ritonavir	Antiviral	CYP3A4	(Ernest *et al*., 2005)
Rofecoxib	Non-steroidal anti-inflammatory drug	CYP1A2	(Karjalainen *et al*., 2006)
Saquinavir	Antiviral	CYP3A4	(Ernest *et al*., 2005)
Ticlopidine	Antithrombotic	CYP2B6 and CYP2C19	(Ha-Duong *et al*., 2001; Richter *et al*., 2004; Turpeinen *et al*., 2005a; Obach *et al*., 2007)
Troleandomycin	Antibacterial	CYP3A4	(Franklin, 1991; Periti *et al*., 1992)
Verapamil	Cardiovascular	CYP3A4	(Ma *et al*., 2000; Wang *et al*., 2004)
Zileuton	Antiasthmatic	CYP1A2	(Lu *et al*., 2003)
Zolpidem	Hypnotic	CYP3A*	(Polasek *et al*., 2010)

* A relatively small effect *in vivo* in humans (Palovaara *et al*., 2000; Polasek *et al*., 2010).

3.2. Chemical mechanisms, kinetics and criteria

Mechanism-based inhibition of CYP enzymes can result from an irreversible modification of the haem, the protein or both the haem and the protein. The modification can occur by the following chemical mechanisms (Zhang *et al*., 2008):

1) the inhibitor binds covalently to the enzyme apoprotein (e.g., CYP3A4 inactivation by grapefruit juice constituents)
2) the inhibitor binds covalently to the prosthetic haem (e.g., CYP3A4 inactivation by gestodene) or
3) the inhibitor chelates or coordinates with the haem (e.g., CYP3A4 inactivation by macrolide antibiotics (Mayhew *et al*., 2000)).

Many mechanism-based inhibitors may inactivate their victim enzymes by more than one mechanism (Kent *et al*., 2001). Chemical structures associated with mechanism-based inhibition include, e.g., substituted imidazoles, furan rings, thiophenoles and acetylenes (Rock, 2008). The kinetics between the enzyme and the inhibitor in mechanism-based inhibition can be described as presented in Figure 3.

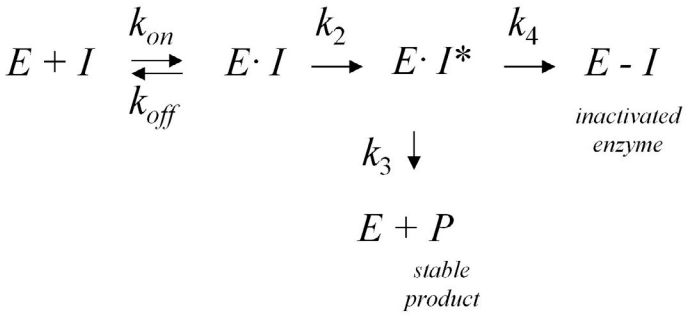

Figure 3. Kinetics between enzyme (E) and inhibitor (I) in mechanism-based inhibition. The initial step that combines the inhibitor and the free enzyme is reversible. The micro constants k_{on}, k_{off}, k_2, k_3 and k_4 describe the reaction velocities.

Mechanism-based inhibition can be described by the kinetic constants maximal inactivation rate (k_{inact}) and the inactivation constant (K_I). The ratio of the end product release to enzyme inactivation is termed as the partition ratio (r) (Walsh *et al*., 1978). For mechanism-based inhibition, it can be described as k_3/k_4. The most dedicated mechanism-based inhibitor would therefore have a partition ratio of 0, that is, every turnover of the inhibitor would produce an inactivated enzyme (Kalgutkar *et al*., 2007).

The criteria for mechanism-based inhibition were set in the 1990's (Silverman, 1995):

1) time-dependency
2) saturation
3) substrate protection
4) irreversibility
5) inactivator stoichiometry

6) involvement of a catalytic step
7) inactivation prior to release of active species.

Since their introduction, the criteria have been specified and argued based on later research results. The current understanding of the criteria for mechanism-based inhibition is presented below:

1) **Time-dependency**. The inhibitory effect of the compound becomes larger when increasing the *in vitro* incubation time or prolonging the dosing period *in vivo*. It is important to note that time-dependent inhibition and mechanism-based inhibition are not synonyms; mechanism-based inhibition is just one form of time-dependent inhibition. Time-dependent inhibition can also occur due to other mechanisms, e.g., due to the formation of metabolites with a stronger inhibitory potential than the parent compound has or due to the so called slow binding inhibition (Zhang *et al*., 2008).

2) **Saturation**. The rate of inactivation increases when the inhibitor is added, until all enzyme molecules are saturated. After that, no further increase in the inactivation rate can be observed, i.e., saturation kinetics of the inactivation can be seen. This phenomenon has later been nominated as pseudo first-order kinetics (Zhou *et al*., 2005b).

3) **Substrate protection**. As mechanism-based inhibitors bind to the active site of the enzyme, the addition of substrates or competitive reversible inhibitors binding to the same site will protect the enzyme from inactivation. This criterion has later been questioned by the findings that alternate substrates in some cases accelerate, rather than protect, the inactivation of the victim enzyme (Masubuchi *et al*., 2002; Polasek *et al*., 2006).

4) **Irreversibility**. The enzyme activity cannot be restored by dialysis or filtration. The criterion of irreversibility is fulfilled at least by the mechanism-based inhibitors producing a covalent bond to the enzyme.

5) **Inactivator stoichiometry**. The number of inactivator molecules to inactivated enzymes should show a 1:1 stoichiometry, i.e., one inactivator molecule should be attached per enzyme active site. However, this criterion is not fulfilled in the case of multimeric enzymes with nonfunctional catalytic sites.

6) **Involvement of a catalytic step**. The unreactive compound needs first to be catalysed by the victim enzyme into a reactive form which is able to destroy the enzymatic activity. The involvement of the catalytic step and the fact that the inhibitor must therefore be acceptable by the enzyme as a substrate may be one reason for the suggestion that mechanism-based inhibitors may exhibit stronger enzyme specificity than reversible inhibitors. Other inactivating compounds being reactive already before a catalytic step by the victim enzyme will not fulfil this criterion, and are therefore not considered mechanism-based inhibitors.

7) **Inactivation prior to release of active species**. The enzyme inactivation must occur prior to the release of the active species. It must be shown that the active species produced by the enzyme does not first dissociate from the enzyme and then return to inactivate the enzyme.

In addition to these 7 original criteria by Silverman, mechanism-based inhibitors of CYP enzymes need to fulfil also the following criteria:

8) **NAPDH-dependent inactivation**. The co-factor needed in CYP catalysed reactions, reduced nicotinamide adenine dinucleotide phosphate (NADPH) is needed also for the inactivation. The system needs to be catalytically competent, and metabolism needs to be occurring (Rock, 2008).

9) **Lack of protection by exogenous nucleophiles / reactive oxygen species scavengers**. The inactivation is not protected by exogenous nucleophiles (e.g., thiols, amines or cyanide) or reactive oxygen species scavengers (e.g., reduced glutathione, catalase or superoxide dismutase) (Fontana *et al*., 2005).

4. Evaluation of enzyme inhibition in drug development

In drug development, the prediction of drug-drug interactions in clinical use is essential (Pelkonen *et al*., 2005). *In vitro* testing of the inhibition of drug metabolism has extensively been used for the prediction of clinical drug-drug interactions since the mid-1990s (Polasek and Miners, 2007).

The kinetic values obtained from the *in vitro* studies of a chemical entity and an understanding of the inhibitor concentrations in clinical use ([I]) form the basis for this *in vitro – in vivo* extrapolation (IVIVE) process for interactions based on enzyme inhibition (Ito *et al*., 2002). The most relevant concentration for the inhibition of hepatic drug metabolism would be the unbound concentration of the inhibitor in the hepatocytes at the enzyme site (Pelkonen and Turpeinen, 2007). Due to ethical reasons, this information is not achievable. Due to the time needed for the drug to reach an equilibrium between blood and hepatocytes, as well as a possible active influx or efflux transport of the drug the hepatic drug concentrations may well differ from drug concentrations in plasma (Ito *et al*., 1998). However, some concentration is needed for the extrapolations. At least average plasma concentration (C_{avg}), maximum plasma concentration (C_{max}), estimated maximum concentrations at the hepatic inlet and more complicated constructs of inhibitor concentration changing over time have been used as surrogates of inhibitor concentration at the enzyme site (Ito *et al*., 1998; Kanamitsu *et al*., 2000b; Tonn *et al*., 2009; Zhang *et al*., 2009). The use of estimated free portal vein C_{max}, especially by eliminating false negative predictions, has worked best for reversible inhibitors, while the use of free systematic C_{max} has provided the best estimates for mechanism-based inhibitors (Obach *et al*., 2007; Obach, 2009). In addition to the issues related to the selection of the concentration used, the impact of plasma protein binding in IVIVE is controversial.

In vitro inhibition cocktail assays have been developed for a rapid screening of the inhibition of major human CYPs (Testino and Patonay, 2003; Walsky and Obach, 2004; Turpeinen *et al*., 2005b; Tolonen *et al*., 2007; Sevior *et al*., 2010). The results from these assays can be used to guide further decisions on the fate of the compounds (Turpeinen *et al*., 2005b; Lahoz *et al*., 2008). However, standard approaches are required to confirm these results.

4.1. Prediction of the clinical importance

The assessment of the inhibition potential for reversible inhibitors is based on the $[I]/K_i$ ratio (Tucker *et al*., 2001). A value of < 0.1 usually indicates a low risk of interaction and a value of > 1 indicates a high risk as shown in Figure 4 (Ito *et al*., 2004).

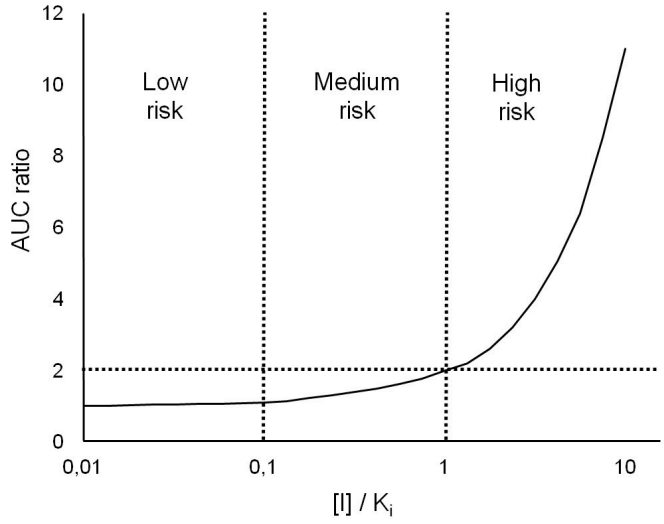

Figure 4. Assessment of inhibition potential of reversible inhibitors based on the $[I]/K_i$ ratio.

Based on the $[I]/K_i$ ratio, the *in vivo* AUC ratio can be predicted also by using the following equation:

$$\frac{AUC_i}{AUC_c} = 1 + \frac{[I]}{K_i}$$

where AUC_i is the area under concentration-time curve in inhibited phase and AUC_c is the area under concentration-time curve in control phase. In order to forecast the interaction potential between the reversible inhibitor and the substrate detailed information is required concerning the substrate metabolism by the enzyme. This can be expressed as the fraction metabolised by the inhibited enzyme (f_m). With the use of substrate specific f_m the predictions of clinical drug-drug interactions have been significantly improved. The f_m can be estimated by several methods. For those drugs which are metabolised by polymorphically expressed CYPs such as CYP2C9, CYP2C19 and CYP2D6, pharmacokinetic studies can be carried out in subjects representing various genotypes. For drugs metabolised by other than polymorphically expressed enzymes, radiolabelled drug metabolism studies and metabolism studies with specific enzyme inhibitors can be performed. Obtaining the f_m can be difficult, and the reliability of the obtained values can be questionable due to considerable interindividual variability (Venkatakrishnan *et al*., 2007).

The estimates of the f_m can provide guidance related to the need of further studies. If human *in vivo* data indicate that CYP enzymes contribute > 25% to the total clearance of a drug, further

studies are needed (Huang *et al*., 2008). In order to achieve an accurate prediction of the interaction potential the evaluation of the metabolism of a new chemical entity should be continued as long as the sum of the contribution rates of each elimination pathway is almost one (typically more than 0.7) (Hisaka *et al*., 2010).

The drug-drug inhibition potential of a reversible inhibitor can be estimated by the following basic equation taking the f_m into account (when the unbound fraction of the victim drug is low):

$$\frac{AUC_i}{AUC_c} = \frac{1}{\frac{f_m}{\left(1+\frac{[I]}{K_i}\right)} + (1 - f_m)}$$

For drugs with considerable intestinal metabolism (e.g., CYP3A4 substrate drugs), the incorporation of intestinal enzyme inhibition improves the predictions. However, the estimation of the inhibitor concentrations at the enzyme site in the gut ($[I]_g$) is difficult. Furthermore, since drugs are frequently metabolised by several CYPs, the metabolism may shift to alternative metabolic pathways, when a single CYP is inhibited. The incorporation of parallel pathways of metabolism has been shown to improve the quantitative predictions of drug-drug interactions based on *in vitro* data (Ito *et al*., 2005; Brown *et al*., 2006). Several important factors are needed to take into account, when the clinical importance of drug-drug interactions is predicted (Table 4).

Table 4. Examples of aspects to be considered in IVIVE of drug-drug interactions.

Inhibitor related aspects	*In vitro* kinetic constants of the inhibitor Inhibitor concentration used in the predictions Changes of inhibitor concentration over time Active transport Parallel interaction mechanisms Inhibitory/inductive metabolites Protein binding
Substrate related aspects	Substrate f_m Parallel pathways of metabolism Protein binding Extraction ratio Therapeutic index
Experimental design related aspects	*In vitro* system used *In vitro* to *in vivo* system differences
Target dosage related aspects (both inhibitor and substrate)	Doses Routes of administration Duration of treatments Timing and order of administration
Target population related aspects	Interindividual variability Age-related differences Ethnic differences Concomitant diseases Concomitant treatments

Predictions made by the basic IVIVE techniques presented above can be refined by physiologically based pharmacokinetic (PBPK) modelling. With PBPK modelling, e.g., the changes in drug concentrations over time *in vivo*, and the extent of bioavailability across the intestinal wall (F_G) can be taken into account (Obach *et al*., 2006; Wang, 2010; Rowland Yeo *et al*., 2011). In addition, models taking several interaction mechanisms simultaneously into account have been developed (Rowland Yeo *et al*., 2011). However, the prediction of interactions of a compound causing multiple effects may be difficult, at least because of the uncertainty of the relationships of the mechanisms *in vivo* (Fahmi *et al*., 2008; Fahmi *et al*., 2009).

Currently, the most advanced techniques of IVIVE perceive also the population variability, e.g., in CYP enzyme expression and activity, in the predictions, and are therefore recommended in drug-drug interaction risk assessment. PBPK modelling is not considered to replace clinical studies, but can now already be used to design these studies. With PBPK modelling the number of clinical studies and subjects participating in them can be reduced. This is particularly relevant for certain populations such as children and patients with renal impairment. With successful PBPK modelling clinical drug-drug interaction studies may become confirmatory rather than exploratory, as was in the past. PBPK modelling is currently a rapidly developing research area. In addition to the variability in the population in general, specific aspects, e.g., ethnicity, specific pharmacokinetic features of children and patients with renal or liver impairment can be taken into account in the modelling of pharmacokinetics and drug-drug interactions (Rostami-Hodjegan and Tucker, 2007; Grime *et al*., 2009; Jamei *et al*., 2009a; Jamei *et al*., 2009b; Johnson *et al*., 2009; Johnson *et al*., 2010; Johnson and Rostami-Hodjegan, 2010).

5. Specific features of the evaluation of mechanism-based inhibition

In addition to the principles presented above, for mechanism-based inhibition the maximal inactivation rate by the inhibitor (k_{inact}) and the first-order turnover degradation rate of the inhibited enzyme (k_{deg}) are essential for successful IVIVE. In fact, in the case of IVIVE using only the values sufficient for reversible inhibition, the interactions based on mechanism-based inhibition can be seriously underestimated, or even failed to be recognised (Venkatakrishnan and Obach, 2007).

In basic *in vitro* inhibition tests, the changes in the kinetic constants V_{max} and K_m in the case of mechanism-based inhibition are similar to the changes seen with reversible noncompetitive inhibitors (Table 2). Therefore, based on data obtained from *in vitro* studies designed for the assessment of reversible inhibition, mechanism-based inhibitors can be incorrectly referred to as reversible noncompetitive inhibitors (Lin and Lu, 1998).

The experiments needed to define the essential inactivation constants are laborious, and therefore, in early drug development with many possibilities of failures and cessation of further development, a step-wise approach in the assessment of the interaction potential is considered reasonable.

5.1. IC_{50} shift assays

Traditionally, the first-line screening methods of mechanism-based inhibition include the demonstration of a time-dependent shift in the concentration supporting half the maximal inhibition (IC_{50}) *in vitro* (Lim *et al*., 2005; Obach *et al*., 2007). In this method, an enhancement of the inhibitory potency (i.e., a decrease in the IC_{50}) is shown to occur during pre-incubation with human liver microsomes (or specific recombinant CYP isoforms) and NADPH prior to incubation with the enzyme-selective index substrate. The incubation process will result in a leftward shift in the IC_{50} curve as shown in Figure 5. The IC_{50} curves of reversible inhibitors without and with preincubation will remain distinguishable, or even show a rightward shift due to the consumption of the inhibitor during the preincubation (Venkatakrishnan and Obach, 2007). It is important to note that a decrease in the IC_{50} shows just time-dependent inhibition, but does not indicate the compound to be a mechanism-based inhibitor. Methods for showing the IC_{50} shift easily, e.g., with only two different preincubation times have been developed (Perloff *et al*., 2009).

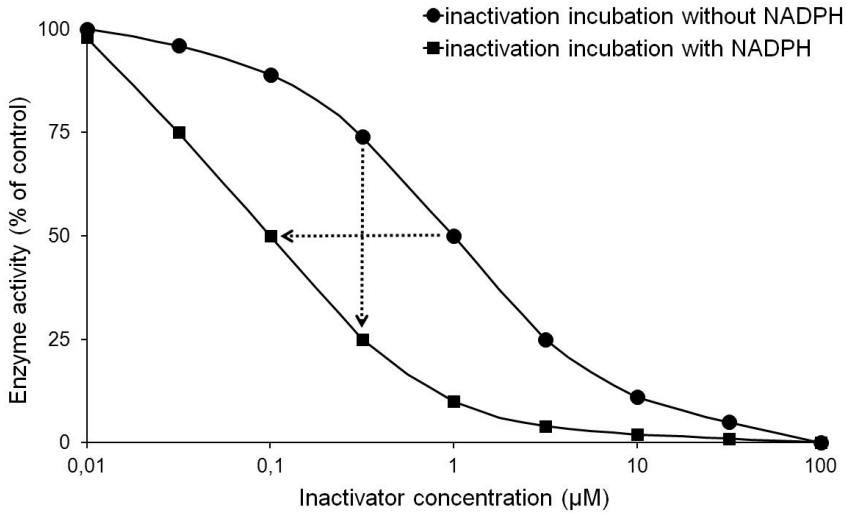

Figure 5. The principle of IC_{50} shift assays for showing time-dependent enzyme inhibition. The enzyme activity with different inhibitor concentrations with incubation without NADPH (black dots) and with incubation with NADPH (black squares) is plotted against the inhibitor concentration. A lower enzyme activity is observed with the same inhibitor concentration with incubation with NADPH compared to that without NADPH (vertical arrow). The concentration supporting half of the maximal inhibition (IC_{50}) decreases with incubation with NADPH compared to that without NADPH (horizontal arrow).

The probe substrate used has been shown not to significantly affect the results of the *in vitro* assessment of mechanism-based inhibition, at least for CYP3A (Watanabe *et al*., 2007). The *in vitro* system, however, which is used for the evaluation of time-dependent inhibition, may affect the results for at least some compounds. Human liver microsomes and recombinant CYPs (expressed in *E. coli*) were not equivalent enzyme sources when the time-dependency of the inhibition of CYP enzymes by tricyclic antidepressants were examined. Nortriptyline showed a time-dependent inhibition in the recombinant CYP2C19 and CYP3A4, but not of the enzymes of human liver microsomes (Polasek and Miners, 2008).

In addition to the demonstration of the IC_{50} shift, techniques of covalent-binding assays with radiolabelled drugs and progress curve analysis methods can be used for a rapid screening of mechanism-based inhibition in early drug development (Evans *et al*., 2004; Salminen *et al*., 2011).

5.2. Kinetic constants

After an observed leftward shift in the IC_{50} curves, *in vitro* tests for the calculation of the kinetic constants are usually carried out. The experimental approach is to perform a preincubation of the enzyme together with co-factors and with different inhibitor concentrations for varying times, followed by a further incubation of a dilution of the reaction mixture with a probe substrate to assess the degree of enzyme inactivation (Grimm *et al*., 2009). A dilution of the reaction mixture at least in the ratio of 1:10 is performed in order to prevent a further inactivation of the enzyme during the probe substrate incubation and to minimise reversible inhibition. If the inhibitor is also a potential reversible inhibitor, more than 20-fold dilution is needed (Grimm *et al*., 2009). The concentration of the probe substrate should approach saturation ($\geq$ 4-fold K_m) (Grimm *et al*., 2009). In addition, the incubation time should be clearly shorter than the preincubation time, to minimise the possibility of reversible or further irreversible inhibition.

From the data obtained, several different plots can be drawn and kinetic constants calculated (Ghanbari *et al*., 2006). The basic graphical plots for mechanism-based inhibition are those presenting the natural logarithm of the remaining enzyme activity against the preincubation time. Separate plots for different inhibitor concentrations are drawn. The apparent rates of inactivation (k_{obs}) can be estimated graphically from the slopes (Figure 6A).

A double reciprocal plot of the obtained k_{obs} against the inhibitor concentration can be used for the calculation of the k_{inact} and K_I (Figure 6B). Alternatively, a Kitz-Wilson plot of the half-life of the inactivation against the inhibitor concentration can be used (Alexander *et al*., 1963; Kitz and Wilson, 1963).

The constants k_{inact} and K_I have been shown not to correlate between each other, indicating that both time of exposure and inhibitor concentration are independent determinants of enzyme inactivation (Zhou *et al*., 2005b). The relationships between k_{obs}, k_{inact} and K_I are shown in Figure 6C.

The partition ratio, an indicator of the efficiency of a mechanism-based inhibitor, can be estimated from a plot with the remaining enzyme activity against the molar ratio of the inhibitor to the enzyme. An estimation of the partition ratio (r) can be calculated from the point of the intercept (number of turnovers, including the inactivation, in the case of 1:1 stoichiometry) (Figure 6D).

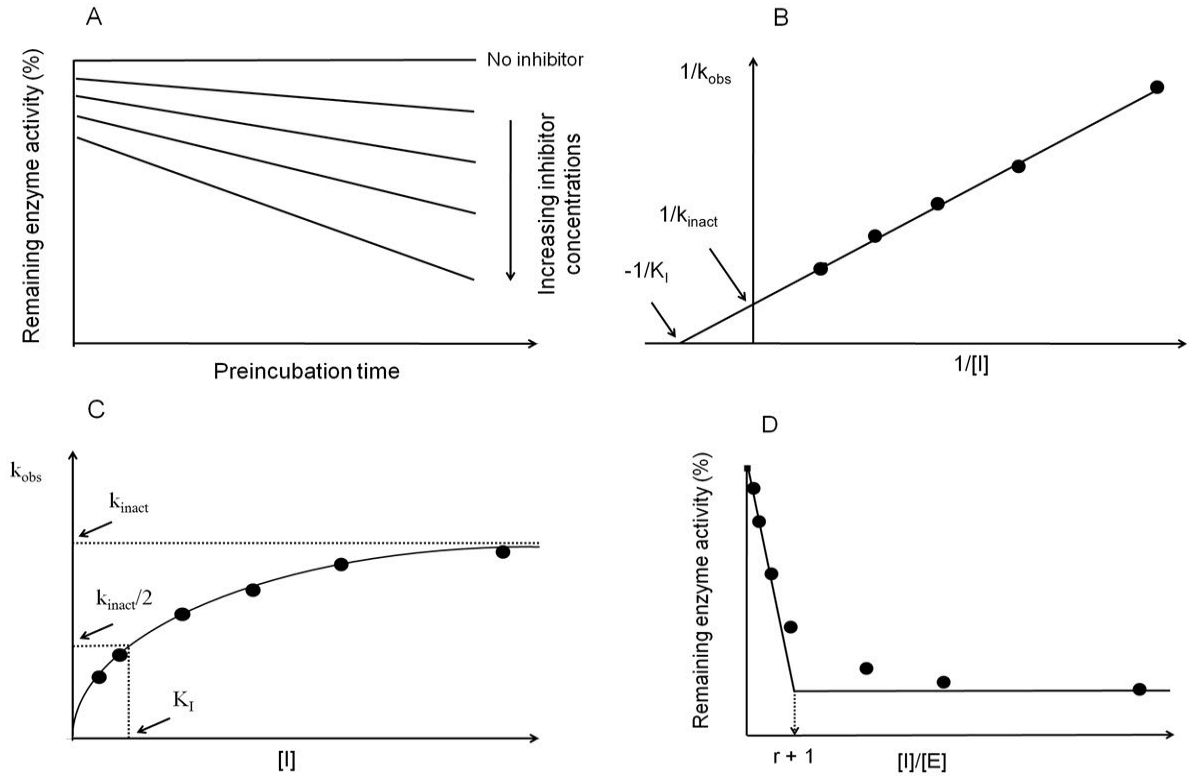

Figure 6. A) Plots of percentage of the remaining enzyme activity (ln) versus preincubation time. B) Double-reciprocal plot of k_{obs} versus inhibitor concentration. C) The relationships between k_{obs}, k_{inact} and K_I. D) Plot for the estimation of the partition ratio (r).

The constants presented above serve as parameters of inactivator efficiency. In addition, the ratio of k_{inact}/K_I may serve as an additional indicator of it (Polasek *et al*., 2004).

Inactivation kinetic constants of some drugs which are mechanism-based inhibitors of CYP enzymes are presented in Table 5.

Table 5. Inactivation kinetic constants of mechanism-based inhibitors.

Inhibited enzyme	*Inhibitor drug (probe drug)*	k_{inact} *(1/min)*	K_I *(μM)*	*Partition ratio*	*Reference*
CYP1A2	Isoniazid	0.11	285		(Wen *et al*., 2002b)
	Rofecoxib	0.07	4.8		(Karjalainen *et al*., 2006)
	Zileuton	0.035	117		(Lu *et al*., 2003)
CYP2A6	Isoniazid	0.13	173		(Wen *et al*., 2002b)
CYP2B6	Clopidogrel	1.5	1.1		(Richter *et al*., 2004)
	Ticlopidine	0.8	0.8		(Richter *et al*., 2004)
CYP2C8	Gemfibrozil 1-O-β-glucuronide	0.21	20		(Ogilvie *et al*., 2006)
	Gemfibrozil 1-O-β-glucuronide	0.072	29	53	(Baer *et al*., 2009)
CYP2C19	Clopidogrel	0.35	0.5		(Richter *et al*., 2004)
	Clopidogrel	0.056	14.3		(Nishiya *et al*., 2009)
	Isoniazid	0.09	112		(Wen *et al*., 2002b)
	Ticlopidine	0.192	87	26	(Ha-Duong *et al*., 2001)
	Ticlopidine	0.5	0.2		(Richter *et al*., 2004)
	Ticlopidine	0.097	4.3		(Obach *et al*., 2007)
	Ticlopidine	0.074	3.32		(Nishiya *et al*., 2009)
	Omeprazole	0.046	9.1		(Paris *et al*., 2008)
CYP2D6	Paroxetine	0.17	0.315*		(Venkatakrishnan and Obach, 2005)
CYP3A4	Diltiazem	0.17	2.2		(Jones *et al*., 1999)
	Diltiazem	0.07	3.3	19.7	(Rowland Yeo and Yeo, 2001)
	Clarithromycin	0.072	5.49		(Mayhew *et al*., 2000)
	Delavirdine	0.59	21.6	41	(Voorman *et al*., 1998)
	Diltiazem	0.027	0.77		(Mayhew *et al*., 2000)
	Erythromycin (midazolam)	0.036	10		(Obach *et al*., 2007)
	Erythromycin (testosterone)	0.039	9.8		(Obach *et al*., 2007)
	Ethinylestradiol			120	(Guengerich, 1988)
	Ethinylestradiol	0.04	18	50	(Lin *et al*., 2002)
	Fluoxetine	0.017	5.26		(Mayhew *et al*., 2000)
	Gestodene	0.4	46	9	(Guengerich, 1990)
	Imatinib	0.072	14.3		(Filppula *et al*., 2011a)
	Isoniazid	0.08	228		(Wen *et al*., 2002b)
	Lapatinib	0.020	1.71	50.9	(Teng *et al*., 2010)
	Mibefradil	0.4	2.3	1.7	(Prueksaritanont *et al*., 1999)
	Ritonavir (midazolam)	0.45	0.38		(Obach *et al*., 2007)
	Ritonavir (testosterone)	0.28	0.18		(Obach *et al*., 2007)
	Verapamil	0.15	2.9	10.3	(Rowland Yeo and Yeo, 2001)
	Verapamil (midazolam)	0.043	1.8		(Obach *et al*., 2007)
	Verapamil (testosterone)	0.043	1.7		(Obach *et al*., 2007)
	Zolpidem	0.094	122		(Polasek *et al*., 2010)
CYP3A5	Fluticasone	0.027	16	3	(Murai *et al*., 2010)

* unbound K_I

5.3. Enzyme degradation rate

The enzyme synthesis rate is generally assumed to be a zero-order process (occurs at the same rate regardless of the concentration), whereas the rate of degradation is a first-order process (occurs at a rate dependent on the concentration) (Yang *et al*., 2008). The first-order rate constant characterising the *in vivo* turnover / degradation of the CYP enzyme (k_{deg}) is essential for the IVIVE of interactions caused by mechanism-based inhibition. The enzyme turnover half-life can be calculated from the first-order degradation rate by the equation half-life ($t_{1/2}$) = $\ln 2/k_{deg}$. The enzyme degradation rate is a physiological value depending on the enzyme, individual and species concerned. As the half-lives of, e.g., rat CYPs are shorter than their human counterparts, their use in IVIVE can under-predict the risk and consequences of mechanism-based inhibition. Therefore, it would be best to use human CYP *in vivo* degradation rates for the IVIVE of human drug interactions. The human CYP *in vivo* k_{deg} values cannot be measured directly. Therefore, several approaches have been used to obtain reliable estimates of them.

In vitro methods for estimating the turnover half-life of human CYPs have been used extensively. Direct measurement of the turnover half-life, e.g., by the pulse-chase method, or rates of loss of the apoprotein contents and enzymatic activity from liver slices or primary cultured hepatocytes have been used, but they do not necessarily provide reliable results due to the loss of *in vivo* conditions. In addition, *in vitro* induction in human hepatocytes can be used for the estimations (Yang *et al*., 2008).

Estimates of human *in vivo* CYP enzyme half-lives can also be achieved by indirect human *in vivo* methods. One method is to analyse the reversal of increased enzyme activity after induction. The recovery of the enzyme activity to basal level is a function of enzyme turnover and any persistence of the inductive effect of the inducer. Therefore, in this method, the inducer should preferably have a short half-life. In addition, the probe substrate used for assessing the enzyme activity should have a short half-life and be extremely specific for the enzyme concerned. In the method utilising mechanism-based inhibition, the degradation half-life is estimated based on the recovery of the enzyme activity to basal level after inactivation. The inhibitor should have a much shorter half-life compared to the enzyme concerned to yield accurate predictions (Yang *et al*., 2008). In addition, pharmacokinetic modelling of CYP autoinduction can be used in the estimation of enzyme turnover (Yang *et al*., 2008).

The turnover half-life for intestinal CYPs can be estimated *in vivo* by inhibitors in doses that inactivate only the intestinal CYP and leave the hepatic CYP unchanged. E.g., the intestinal CYP3A4 turnover half-life has been estimated at 23 h by using single doses of grapefruit juice as the inhibitor (Greenblatt *et al*., 2003). However, as the process for enterocyte maturation is probably faster than enzyme turnover, intestinal CYP half-lives are likely to be determined by cell rather than enzyme turnover (Yang *et al*., 2008).

Human CYP enzymes can be divided into two groups based on their estimated degradation rates, most of which have been yielded by *in vitro* methods. CYP2C9, CYP2D6, CYP3A4 and CYP4A11 can be considered relatively stable (half-lives between 70 and 104 h), compared to

CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP2E1 and CYP3A5, which were considered relatively unstable (half-lives between 23 and 36 h) (Renwick *et al*., 2000).

The sensitivity of the predicted drug-drug interaction to the enzyme degradation rate used has been studied at least for CYP2D6 and CYP3A4. Not surprisingly, the predictions were most sensitive to different k_{deg} values when the f_m values of the substrate were the largest (Venkatakrishnan and Obach, 2005; Galetin and Houston, 2006).

5.4. *In vitro – in vivo* extrapolation

The prediction of the clinical importance of mechanism-based inactivation based on *in vitro* studies is more complicated than that of reversible inhibition. In addition to the $[I]/K_I$ ratio, the k_{inact}/k_{deg} ratio is an independent factor in the inhibition potential. Therefore, even if the $[I]/K_I$ ratio is very small, the clinical interaction can be relevant in case of very rapid inactivation making the k_{inact}/k_{deg} ratio sufficiently large (Venkatakrishnan and Obach, 2007).

The following equation can be used for the extrapolation of the *in vivo* potency of a mechanism-based inhibitor:

$$\frac{k_{inact} \cdot [I]}{k_{deg}} \cdot (K_I + [I])$$

If the obtained index is << 1, a weak inhibition can be expected, with a victim drug AUC fold increase of ≤ 2. The opposite applies to moderate and potent inhibition (Zhou and Zhou, 2009).

The clinical impact of mechanism-based inhibition on CYP-mediated clearance can be predicted from *in vitro* inactivation data using the following mathematical model, which takes the importance of the inactivated enzyme to the substrate metabolism into account (Mayhew *et al*., 2000; Jones and Hall, 2002):

$$\frac{AUC_i}{AUC_c} = \frac{1}{\frac{f_m}{1 + \left(\frac{k_{inact} \cdot [I]}{k_{deg} \cdot (K_I + [I])} \right)} + (1 - f_m)}$$

If the inhibitor concentration ([I]) is below K_I, a simplified version of the equation presented above can be used:

$$\frac{AUC_i}{AUC_c} = \frac{1}{\frac{f_m}{1 + \left(\frac{k_{inact} \cdot [I]}{k_{deg} \cdot K_I} \right)} + (1 - f_m)}$$

These mathematical models are based on a number of simplifying assumptions: 1) the substrate drug is well absorbed and the inhibitor has no effect on its absorption, 2) the substrate exhibits linear pharmacokinetics and is metabolised by the liver only, 3) the hepatic clearance of the substrate can be described by the well stirred model and 4) the degradation rate of the enzyme is a first order process and the inhibitor has no effect on it (Lu *et al*., 2003).

The inactivation of the intestinal enzyme is relevant for at least predictions of CYP3A4 mediated interactions, and can be taken into account by the following equation (Wang *et al*., 2004):

$$\frac{AUC_i}{AUC_c} = \frac{1}{F_g + (1 - F_g) \cdot \frac{1}{1 + \left(\frac{k_{inact} \cdot [I]_g}{k_{deg,gut} \cdot ([I]_g + K_I)} \right)}} \cdot \frac{1}{\frac{f_m}{1 + \left(\frac{k_{inact} \cdot [I]}{k_{deg,hep} \cdot K_I} \right)} + (1 - f_m)}$$

where F_g is the fraction of the dose of the affected drug that passes through the intestine unchanged after oral administration in the control state, $[I]_g$ is the concentration of the inhibitor in the intestine, $k_{deg,gut}$ is the *in vivo* degradation rate of the enzyme in the intestine and $k_{deg,hep}$ is the *in vivo* degradation rate of the enzyme in the liver.

The selection of the inhibitor concentration to be used in the clinical drug-drug interaction predictions, as discussed already for IVIVE in general, is important also for mechanism-based inhibitors. For mechanism-based inhibitors, the use of C_{max} in the extrapolations has yielded the most accurate predictions of the interaction potential (Obach *et al*., 2007; Obach, 2009; Sato *et al*., 2010). In addition to the issues related to concentration selection, mispredictions may arise due to plasma protein binding, atypical substrate pharmacokinetics, the existence of inhibitory metabolites, the partitioning from plasma to liver, the rate-limiting transport of the substrate and the inhibitor into the hepatocytes, the intestinal active efflux of the substrate and inhibitor as well as the extrahepatic metabolism of both players (Zhou *et al*., 2005b).

Mechanism-based inhibition can be modelled by PBPK modelling techniques. The sorivudine-5-fluorouracil interaction causing fatal outcomes in Japan could be quantitatively predicted later with a PBPK model. Similarly, the interaction between mechanism-based CYP3A4 inhibitor erythromycin and CYP3A4 substrate triazolam was predicted by PBPK model (Kanamitsu *et al*., 2000a; Kanamitsu *et al*., 2000c; Ito *et al*., 2003). The accuracy of the predictions of mechanism-based inhibition by static models and PBPK modelling has been compared. Some results have indicated that static models would provide more accurate predictions, whilst some have shown that PBPK models would be better (Einolf, 2007; Wang, 2010).

5.5. The role of *in vivo* animal studies

Due to the limitations of *in vitro* and *in silico* methods, *in vivo* animal studies (usually in mice and rats) have also been used in the assessment of mechanism-based inhibition. Interspecies variation in many aspects, e.g., in the CYP amino acid sequence, substrate specificity and catalytic activity, however, can cause difficulty in extrapolating animal data to humans, but it is particularly challenging in the case of mechanism-based inhibition (Lin, 1995; Zhou *et al*., 2005b; Sekiguchi *et al*., 2008; Zhang *et al*., 2010). Monkeys have been proposed recently as a suitable model animal to predict drug-drug interactions caused by the mechanism-based inhibition of CYP3A (Ogasawara *et al*., 2009). An approach of combining data from *in vivo* rat studies and *in vitro* studies with human enzymes could also be useful to evaluate risks in clinical studies (Sekiguchi *et al*., 2008). Further, at least for CYP3A inactivation, CYP enzyme humanised mice have been proposed to be used for evaluating mechanism-based inhibition (Aueviriyavit *et al*., 2010).

In spite of rigorous *in vitro* methods, *in vivo* animal studies and advanced IVIVE techniques, clinical drug-drug interaction studies in humans may be needed anyway for the evaluation of the mechanism-based inhibition potential of new chemical entities. Probe substrates for studying the activity of major CYPs involved in drug metabolism in humans have been recommended by the Food and Drug Administration of United States (FDA, 2006).

6. Effects of time and dose on mechanism-based inhibition

Mechanism-based inhibition is both a time- and concentration-dependent phenomenon. Its time and dose relationships have been studied extensively *in vitro*. The time-dependency of inhibition is required to show that an agent is a mechanism-based inhibitor. Concentration-dependency is used to determine the *in vitro* kinetic constants. However, data concerning the time and dose relationships of mechanism-based inhibition *in vivo* in humans is sparse.

6.1. Effects of time on mechanism-based inhibition

Onset of the inhibitory effect. There are only a few publications available regarding the onset of mechanism-based inhibition. *In vitro* and *in vivo* studies examining the onset of the inhibitory effect concern mainly the inactivation of CYP3A4 by grapefruit constituents. The inhibitory effect of grapefruit juice has been shown to have reached its maximum already after the first dose (on day 1), when reflected as changes in felodipine pharmacokinetics on day 1 and day 14, compared to control (Lundahl *et al*., 1998). The onset of the mechanism-based inhibition caused by grapefruit juice furanocoumarins bergamottin and 6',7'-dihydroxybergamottin has been compared *in vitro* using CYP3A4-expressing human colorectal adenocarcinoma derived Caco-2 cells and midazolam and testosterone as probe drugs. The inactivation of CYP3A4 was shown to occur more rapidly by 6',7'-dihydroxybergamottin than by bergamottin. 6',7'-dihydroxybergamottin inhibited the CYP3A4-mediated metabolism of the probe drugs > 85% already in 30 minutes, whereas the CYP3A4 inhibition by bergamottin reached a level of ≥ 70% after 3 h (Paine *et al*., 2005).

The onset of inhibitory effects by ritonavir has recently been studied. The inhibition, measured as a decrease in midazolam clearance, developed to its maximum in 48 h after starting ritonavir 300 mg twice daily (Katzenmeier *et al*., 2011). In addition, physiologically-based pharmacokinetic modelling has been used to predict the onset of mechanism-based inactivation of CYP enzymes. MDMA (ecstasy) was estimated to inactivate CYP2D6 already in 1 h (Yang *et al*., 2006).

Recovery from the inhibitory effect. One characteristic of mechanism-based inhibition is that, in contrast to reversible inhibition, it persists even after the removal of the inhibitor. The inhibition is reversed *in vivo* by *de novo* synthesis of the protein, and the rate of the recovery to basal state is determined by the first-order degradation rate (k_{deg}) / the half-life ($t_{1/2}$) of the inhibited enzyme. The methods for estimating these have been described in section 5.3.

Examples of published data from *in vivo* studies regarding the half-lives / recovery time of clinically relevant CYP enzymes are provided in Table 6.

Table 6. Examples of clinical studies with data on CYP enzyme half-lives / enzyme recovery after mechanism-based inhibition or induction.

CYP enzyme	*Inhibitor*	*Inducer*	*Probe for enzyme activity*	*Results*	*Reference(s)*
CYP1A2	-	Smoking	Caffeine	$t_{½}$ = 39 h	(Faber and Fuhr, 2004)
CYP2C8	Gemfibrozil	-	Repaglinide	Inhibitory effect lasts at least 12 h	(Tornio *et al*., 2008a)
CYP2D6	Fluoxetine, sertraline, paroxetine	-	Dextrometorphan	$t_{½}$ = 70 h, corrected with paroxetine half-life and using PBPK modelling to 51 h	(Liston *et al*., 2002; Venkatakrishnan and Obach, 2005)
	MDMA (ecstasy)	-	Dextrometorphan	$t_{½}$ = 47 h, recovery takes 10 days	(O'Mathuna *et al*., 2008)
CYP2E1	-	Alcohol	Chlorzoxazone	$t_{½} \approx$ 60 h	(Lucas *et al*., 1995)
	Disulfiram	-	Chlorzoxazone	$t_{½}$ = 50 h	(Emery *et al*., 1999)
CYP3A4	Grapefruit juice	-	Felodipine	Inhibitory effect lasts at least 24 h; $t_{½}$ = 8 h	(Lundahl *et al*., 1995; Takanaga *et al*., 2000a)
	Grapefruit juice	-	Nisoldipine	Recovery takes > 3 days	(Takanaga *et al*., 2000b)
	Grapefruit juice	-	Simvastatin	Recovery takes 3-7 days	(Lilja *et al*., 2000b)
	Grapefruit juice	-	Midazolam	$t_{½}$ = 23 h (intestinal CYP3A4)	(Greenblatt *et al*., 2003)
	Grapefruit juice (acute and extended exposure), ritonavir	-	Triazolam	Recovery takes 3 days	(Culm-Merdek *et al*., 2006)
	Ritonavir	-	Midazolam	Inhibitory effect lasts at least 3 days	(Katzenmeier *et al*., 2011)

6.2. Effects of dose on mechanism-based inhibition

It is generally accepted that the *in vivo* inhibitory effect of mechanism-based inhibitors is based on the k_{inact}, K_I, k_{deg} and the dose (concentration of the inhibitor at the enzyme active site). The dose-dependency of inhibition of drug metabolism has been reported for some reversible and irreversible inhibitors. However, human *in vivo* studies on the effect of the dose on mechanism-based inhibition are quite sparse (Levy *et al*., 2003).

Examples of *in vivo* human studies providing results on the dose-dependency of the interactions caused by agents known to be mechanism-based inhibitors of CYP enzymes are provided in Table 7. Studies concerning weak mechanism-based potential are excluded. It should be noted that the compounds may also have other mechanisms than mechanism-based inhibition leading to changes in the substrate concentrations. E.g., the substrate drug may be metabolised by several enzymes. The other mechanisms leading to the changes in substrate concentrations may include various factors such as additional reversible inhibition or induction of the same enzyme; inhibition and induction of other enzymes; and inhibition and induction of transporter proteins contributing to the substrate drug's disposition. Therefore, these results concerning dose-dependency may not be related to mechanism-based inhibition, but reflect the dose-dependency of the total effect of the perpetrator on the clearance of the substrate drug. In some of the studies no dose-dependency could be shown. This may be due to a small number of doses being studied. The inhibition may have been large already at the small inhibitor doses studied, and therefore no significant further increase in the inhibition could be obtained with large doses. In these dose-dependency studies (Table 7), a use of PBPK models was not reported.

Studies designed for assessing the dose-dependency of mechanism-based inhibitors have provided also other interesting results. Bailey *et al*. tried to study the dose-/concentration-dependency of mechanism-based inhibition *in vitro* and *in vivo*. The concentrations of bergamottin in grapefruit and lime juice were made equal by diluting lime juice with water to ¼ strength of the original. The CYP3A4 inhibitory effects of the juices were compared. Grapefruit juice was shown to inhibit CYP3A4 metabolism more than lime juice, indicating that bergamottin is not likely to be the primary substance in grapefruit juice causing mechanism-based CYP3A4 inactivation (Bailey *et al*., 2003). Later, the difference in the inactivation has been detected to be due to 6',7'-dihydroxybergamottin, which is present in grapefruit juice and absent in lime juice (Paine *et al*., 2005).

Veronese *et al*. (2003) have studied the inhibitory potency of two doses of grapefruit juice on CYP3A4 and they have also attempted to determine the major issue of this CYP3A4-inhibition. In that study, double-strength grapefruit juice or water was administered once daily for 2 days, and then, 90, 60 and 30 min before the probe drug (midazolam) on day 3. The study included an additional part in which single doses of normal-strength and double-strength grapefruit juice or water were administered. Double-strength grapefruit juice in multiple doses was shown to increase the AUC, C_{max} and $t_{1/2}$ of midazolam, and decrease the amount of exhaled $^{14}CO_2$ in the erythromycin breath test compared to the control (water) phase, which was considered to reflect the inhibition of both intestinal and hepatic CYP3A4.

Table 7. Examples of *in vivo* human studies providing data concerning the dose-dependency of mechanism-based inhibitors.

Inhibitor	*Inhibitor dosing scheme*	*Substrate(s)*	*Results relating to inhibitor dose*	*Reference*
Cimetidine	1000 or 1600 mg daily for 4 days.	Tolbutamide, antipyrine	1000 mg had no significant effect on tolbutamide or antipyrine elimination; 1600 mg impaired the elimination of both substrates.	(Back *et al*., 1988)
Cimetidine	800 mg or 1200 mg daily for 15 days.	Warfarin	No dose effect shown.	(Sax *et al*., 1987)
Diltiazem	30 or 90 mg three times a day for 3 days.	Nifedipine	The mean AUC of nifedipine increased with both diltiazem doses, the increase was larger with a 90 mg dose (3.1) than with a 30 mg dose (2.2) ($P < 0.05$).	(Tateishi *et al*., 1989)
Diltiazem	10, 20, 30, 60, 120 or 180 mg daily for ≥ 2 weeks.	Tacrolimus	Dose-dependent increases in tacrolimus AUC.	(Jones and Morris, 2002)
Disulfiram	250 mg or 500 mg daily for 4 or 5 days.	Caffeine	No dose effect shown.	(Beach *et al*., 1986)
Disulfiram	250 or 500 mg daily for 8 days.	Theophylline	Decrease in theophylline clearance with 500 mg disulfiram was larger (33%) than with 250 mg (21%) ($P < 0.01$).	(Loi *et al*., 1989)
Grapefruit juice	200 ml of normal-strength juice once, 200 ml of double-strength juice once or 3 times daily for 2 days.	Triazolam	Triazolam concentrations were increased more with double-strength grapefruit juice in the multiple dosing scheme (AUC 2.4-fold) than in the single dosing schemes with double-strength (AUC 1.5-fold) and single-strength (AUC 1.5-fold) juice.	(Lilja *et al*., 2000a)
Paroxetine	10 mg twice daily for 7 days, 20 mg twice daily during the consecutive 7 days.	Nortriptyline	Nortriptyline concentrations were increased more after 20 mg doses (5-6-fold) than after 10 mg doses (3-fold) of paroxetine ($p \leq 0.01$).	(Laine *et al*., 2001)
Ritonavir	100 or 300 mg twice daily for 7 or 14 days.	Amprenavir	No other dose effect of ritonavir shown than adverse events more frequent with 300 mg dose.	(Sadler *et al*., 2001)
Ritonavir	200, 300 or 400 mg twice daily for 14 days.	Saquinavir	400 mg ritonavir increased saquinavir concentrations marginally more than lower doses.	(Buss *et al*., 2001)
Ritonavir	200, 300 or 400 mg twice daily for 15 days.	Indinavir	No dose effect shown.	(Hsu *et al*., 1998)
Ritonavir	100 or 200 mg daily for 14 days.	Nelfinavir	No dose effect shown.	(Kurowski *et al*., 2002)
Verapamil	80 mg three times daily for 3 days, 120 mg three times daily for 3 consecutive days.	Quinidine	No dose effect shown.	(Edwards *et al*., 1987)

In contrast, a single dose of grapefruit juice (either normal or double-strength), increased only the AUC and C_{max} of midazolam, with only minimal effects on midazolam $t_{1/2}$ or the erythromycin breath test, indicating a preferential inhibition of intestinal CYP3A4. In this study, the effects of single doses of normal and double-strength grapefruit juice on midazolam pharmacokinetics or the erythromycin breath test did not differ significantly from each other (Veronese *et al*., 2003).

A proper assessment of human *in vivo* dose-dependency of inhibitory potency requires the incorporation of data concerning the dose-proportionality of the inhibitor. The assessment of the dose-proportionality is also needed for other purposes than the assessment of the inhibitory potency of the compound, and is currently a standardised part of the drug development process. Dose-proportionality assessments are recommended to be done by cross-over methods and power-model statistical approaches, at least if the number of subjects in the pharmacokinetic study is small (Sheng *et al*., 2010).

7. Management and utilisation of mechanism-based inhibition

The finding that a compound is a mechanism-based inhibitor does not necessarily mean that it cannot be used as a therapeutic agent. In fact, many widely used drugs are mechanism-based inhibitors (Jones and Hall, 2002) (Table 5). However, caution should be exercised, when many of these drugs are used clinically. Tools for the management of mechanism-based inhibition include early identification of the drugs behaving as irreversible inhibitors, rational use of such drugs, therapeutic drug monitoring and prediction of risks for potential drug-drug interactions (Zhou *et al*., 2005a). A rational use of mechanism-based inhibitors can be achieved, e.g., by selecting the patients' concomitant drugs carefully, adjusting the dose of the inhibitor or substrate drug and by discontinuing treatment in case of toxic interactions. One additional tool is the development of "hard drugs" which are non-metabolisable and therefore avoid mechanism-based inhibition (Zhou, 2008).

Since the first description of the phenomenon of mechanism-based inhibition of enzymes it has been widely used in enzymology and clinical drug therapy (Osawa and Pohl, 1989; Lin and Lu, 1998). For example the inactivation of xanthine oxidase by allopurinol has been used in the treatment of gout (Massey *et al*., 1970; Silverman, 1988). Other clinical examples include ritonavir, which is frequently used with other protease inhibitors (e.g., saquinavir, lopinavir, indinavir and amprenavir) in the treatment of human immunodeficiency virus infection as it has been shown to enhance their oral bioavailability and to increase their concentrations in plasma (Kurowski *et al*., 2002). This effect is at least partly due to the mechanism-based inactivation of CYP3A4 (Buss *et al*., 2001).

8. Cytochrome P450 2C8

The cytochrome P450 2C8 enzyme (CYP2C8) was purified from the human liver in the 1980's (Lasker *et al*., 1987). It is a key member of the CYP2C family and accounts for approximately 6-7% of the total hepatic CYP content. The role of CYP2C8 in drug metabolism and especially in drug-drug interactions and pharmacogenomics has been recognised only recently (Totah and Rettie, 2005). It has been estimated that CYP2C8 metabolises 5-8% of drugs which are cleared by phase I reactions (Lai *et al*., 2009). Examples of endogenous and exogenous substrates of CYP2C8 are shown in Table 8.

Table 8. Examples of substrates, inhibitors and inducers of CYP2C8.

Substrates	*Reference*	*Inhibitors*	*Reference*
All-*trans*-retinoic acid	(Marill *et al*., 2000)	Amiodarone	(Polasek *et al*., 2004)
Amiodarone	(Ohyama *et al*., 2000)	Clotrimazole	(Ong *et al*., 2000)
Amodiaquine	(Li *et al*., 2002)	Fluoxetine	(Polasek *et al*., 2004)
Arachidonic acid	(Rifkind *et al*., 1995)	Gemfibrozil	(Wang *et al*., 2002)
Chloroquine	(Kim *et al*., 2003)	Gemfibrozil 1-O-β glucuronide	(Ogilvie *et al*., 2006)
Cerivastatin	(Wang *et al*., 2002)	Isoniazid	(Polasek *et al*., 2004)
Fluvastatin	(Prueksaritanont *et al*., 1999)	Ketoconazole	(Ong *et al*., 2000)
Ibuprofen	(Hamman *et al*., 1997)	Montelukast	(Walsky *et al*., 2005)
Loperamide	(Kim *et al*., 2004)	Nortriptyline	(Polasek *et al*., 2004)
Montelukast	(Karonen *et al*., 2010; Filppula *et al*., 2011b)	Quercetin	(Bun *et al*., 2003)
Morphine	(Projean *et al*., 2003)	Trimethoprim	(Wen *et al*., 2002a; Hruska *et al*., 2005)
Paclitaxel	(Rahman *et al*., 1994)	Verapamil	(Polasek *et al*., 2004)
Pioglitazone	(Jaakkola *et al*., 2006)	*Inducers*	*Reference*
Repaglinide	(Bidstrup *et al*., 2003)	Dexamethasone	(Gerbal-Chaloin *et al*., 2001)
Rosiglitazone	(Baldwin *et al*., 1999)	Phenobarbital	(Gerbal-Chaloin *et al*., 2001)
Troglitazone	(Yamazaki *et al*., 1999)	Rifampicin	(Gerbal-Chaloin *et al*., 2001)

The size of CYP2C8 is approximately 2.7 Å and its molecular mass is 53.5 kDa. Its substrate binding cavity is relatively large (1438 $Å^3$) compared to many other human CYPs. Due to its large substrate binding cavity CYP2C8 can accommodate several large substrates and inhibitors, such as paclitaxel and montelukast (Schoch *et al*., 2008). As CYP3A4 also has a large substrate binding cavity (1386 $Å^3$), many substrates of CYP2C8 are also metabolised by CYP3A4 (Lai *et al*., 2009). CYP2C8 exists as dimers in natural membranes (Schoch *et al*., 2004), and this has been proposed to have functional significance (Hu *et al*., 2010).

CYP2C8 is principally expressed in the liver. However, the messenger ribonucleic acid (mRNA) of the *CYP2C8* and/or CYP2C8 protein have been detected in many extrahepatic tissues, e.g., the duodenum, kidney, adrenal gland, heart, lung, brain, mammary gland, uterus and ovary (Klose *et al*., 1999; Läpple *et al*., 2003; Enayetallah *et al*., 2004; Delozier *et al*., 2007; Michaud *et al*., 2010).

The *CYP2C8* gene is located in the chromosome 10q24 near other *CYP2C* genes in the following order from centromere to telomere: Cen-2C18-2C19-2C9-2C8-Tel (Gray *et al*., 1995). The *CYP2C8* gene spanning 31 kb and containing 9 exons is the smallest gene in the CYP2C family (Klose *et al*., 1999). At least nuclear receptors glucocorticoid receptor (GR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are considered to participate in the regulation of *CYP2C8* (Gerbal-Chaloin *et al*., 2001; Lai *et al*., 2009). Since the first report of *CYP2C8* gene polymorphism in 2001 (Dai *et al*., 2001), at least 15 variants of the CYP2C8 gene have been identified to date (http://www.cypalleles.ki.se). At least some of the genetic polymorphisms in *CYP2C8* have functional significance (Daily and Aquilante, 2009). The wild type of the *CYP2C8* gene is named as *CYP2C8*1*. *CYP2C8*2*, encoding a CYP2C8 protein with decreased paclitaxel hydroxylation activity *in vitro*, is expressed in black populations with an allele frequency of 18%, but is very rare in white subjects (allele frequency < 1% in Caucasians) (Dai *et al*., 2001). *CYP2C8*3*, instead, is frequently expressed in white subjects (allele frequency 15%) (Bahadur *et al*., 2002). The functional activity level of the CYP2C8.3 protein encoded by *CYP2C8*3* is currently ambiguous. *CYP2C8*3* has been associated with decreased arachidonic acid and paclitaxel metabolism *in vitro* and for paclitaxel also *in vivo* (Dai *et al*., 2001; Bahadur *et al*., 2002; Lundblad *et al*., 2005; Bergmann *et al*., 2011). In contrast, amiodarone metabolism was not shown less effective with CYP2C8.3 than with CYP2C8.1 (Soyama *et al*., 2002). Furthermore, in *in vivo* studies, *CYP2C8*3* has been associated with increased metabolic activity, e.g., towards repaglinide, rosiglitazone and pioglitazone (Niemi *et al*., 2003c; Kirchheiner *et al*., 2006; Tornio *et al*., 2008b). Several explanations for this *in vitro - in vivo* discrepancy have been proposed, such as compensatory upregulation of other CYP enzymes *in vivo* in subjects carrying *CYP2C8*3* (Niemi *et al*., 2003c). Recently, a new theory of substrate dependent interactions of CYP2C8.3 and cytochrome P450 reductase and cytochrome b5 was proposed (Kaspera *et al*., 2011). The allele frequency of *CYP2C8*4* encoding a protein with conflicting data concerning enzymatic activity, is 7.5% in Caucasians (Bahadur *et al*., 2002). It was also recently suggested that in addition to different metabolic activities, the genetic variants of CYP2C8 may have altered drug inhibitory susceptibilities compared to CYP2C8.1 (Gao *et al*., 2010). Genetic variance in *CYP2C8* has been observed to be associated with the risk of myocardial infarction (Yasar *et al*., 2003). This is considered to be related to the metabolism of arachidonic acid to epoxyeicosatrienoic acids, which are supposed to have a cardiovascular protective role (Rodenburg *et al*., 2010).

In addition to genetic issues, CYP2C8 activity can vary due to various factors such as concomitantly used drugs (Table 8). CYP2C8 activity is determined *in vitro* by using paclitaxel 6α-hydroxylation and amodiaquine N-deethylation assays. Rosiglitazone, retinoic acid, fluvastatin and torsemide have also been suggested as *in vitro* probes for CYP2C8 activity (Melet *et al*., 2004). For *in vivo* studies, repaglinide, rosiglitazone, pioglitazone, cerivastatin and most recently montelukast have been recommended as probe substrates (FDA, 2006; Lai *et al*., 2009; Karonen *et al*., 2011; VandenBrink *et al*., 2011). Also pharmacologically inactive enantiomer of ibuprofen, R-ibuprofen has been proposed to be used as an *in vivo* probe for CYP2C8 activity (Lai *et al*., 2009). However, it seems to be very insensitive to the inhibition of CYP2C8 (Tornio *et al*., 2007).

9. Gemfibrozil and gemfibrozil 1-O-β-glucuronide

Gemfibrozil is a fibric acid derivative developed for the treatment of dyslipidaemia. It was discovered in the 1960's and the first clinical study in healthy volunteers was carried out in 1971 (Smith, 1976). Gemfibrozil was well-tolerated in early studies and it reduced triglyceride levels and increased high-density lipoprotein levels in the subjects. The pharmacological actions of gemfibrozil are mediated mainly through peroxisome proliferator activated receptor alpha (PPARα) stimulation, but its consequences are not completely understood. They are considered to involve at least a stimulation of free fatty acid oxidation, an increase in lipoprotein lipase activity and a decrease in the synthesis of apolipoprotein C-III. The basic characteristics of gemfibrozil are shown in Table 9.

Table 9. Characteristics of gemfibrozil (Okerholm *et al*., 1976; Todd and Ward, 1988; Miller and Spence, 1998; Bersot, 2010).

Chemical structure	5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoicacid, $C_{15}H_{22}O_3$
Molecular weight	250.3
Usual daily dosage	600 mg twice or 900 mg once daily
Oral bioavailability (F)	Almost 100%
$t_{1/2}$	1.5 h
Plasma protein binding	> 99% (mainly to albumin)
Volume of distribution (V_d)	9-13 l
Metabolism	Glucuronidation, phase I reactions
Route of excretion	Renal (66%), biliary (6%)

Gemfibrozil was first marketed in the United States in 1982. Since then, marketing authorisation for gemfibrozil has been granted in many other countries. Based on the results from the Helsinki Heart Study in 1987 gemfibrozil was shown to reduce cardiac endpoint events in patients with primary hyperlipidaemia by 34% compared to placebo. In addition, mortality due to coronary disease decreased by 26%, but there were no differences in the total mortality between the gemfibrozil and placebo groups (Frick *et al*., 1987). In a secondary prevention study, similarly, gemfibrozil decreased both fatal and nonfatal coronary events by 22% (Rubins and Robins, 2000). Based on these promising clinical studies gemfibrozil was considered as well-tolerated in clinical use (Athyros *et al*., 1997; Murdock *et al*., 1999).

In the 1990's reports of increased incidence of rhabdomyolysis in patients treated concomitantly with gemfibrozil and 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) were published (Furberg and Pitt, 2001). About one third of the patients treated with cerivastatin, who developed fatal rhabdomyolysis, had used gemfibrozil concomitantly. Since both gemfibrozil and statins can cause myopathy, a pharmacodynamic interaction was suspected. However, later the plasma concentrations of cerivastatin were shown to be increased by more than 5-fold by gemfibrozil in conjuction with a large reduction in the concentrations of the CYP2C8-dependent major metabolite of cerivastatin (Backman *et al*., 2002). Although the clinical use of gemfibrozil has decreased, it has a role in the management of hyperlipidaemia in certain individuals such as in patients with isolated

hypertriglyceridaemia, diabetes mellitus or metabolic syndrome (Barter and Rye, 2008; Loomba and Arora, 2010) and in the case of intolerance of statin treatment. In addition, the drug-drug interaction potential of gemfibrozil can be utilised both in *in vitro* and *in vivo* studies during drug development. It is of interest that gemfibrozil is currently a recommended model inhibitor of CYP2C8 for *in vitro* and *in vivo* studies by regulatory authorities (FDA, 2006; EMA, 2010).

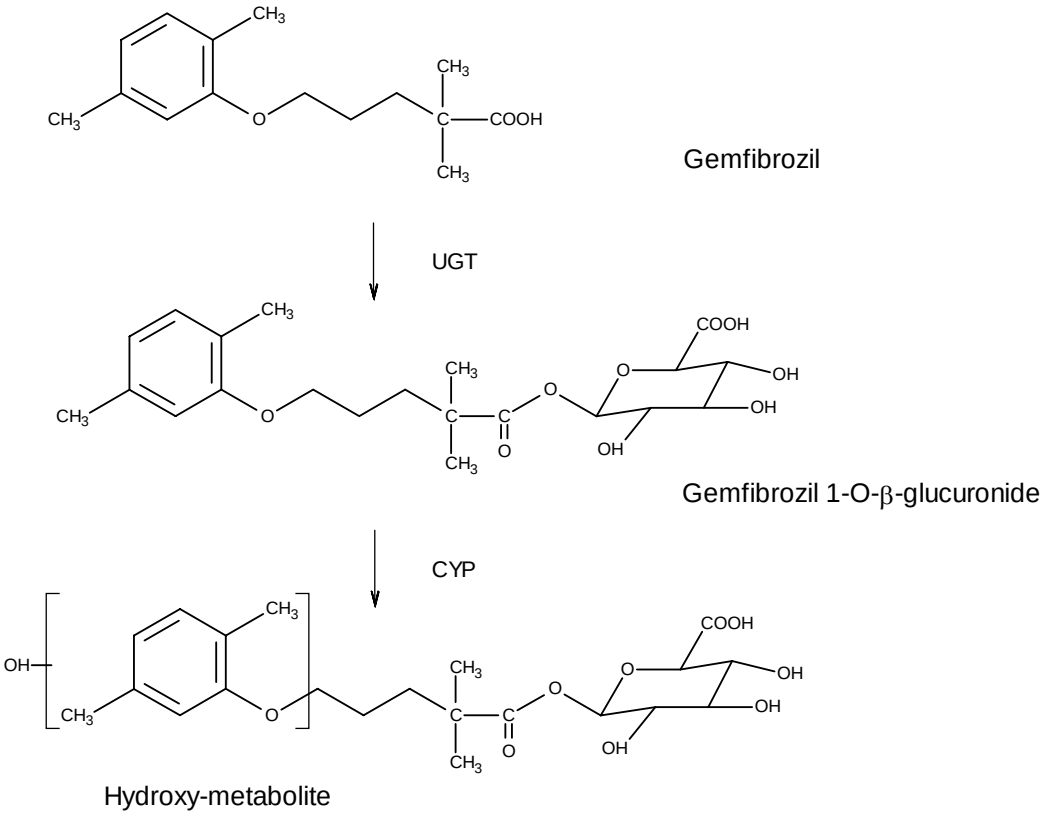

Figure 7. Gemfibrozil 1-O-β-glucuronide formation and metabolism (Ogilvie *et al*., 2006).

9.1. *In vitro* and *in vivo* effects of gemfibrozil and gemfibrozil 1-O-β-glucuronide

Gemfibrozil is glucuronidated to 1-O-β-glucuronide in the hepatocytes mainly by the uridine-5'-diphosphoglucuronosyltransferase (UGT) 2B7 enzyme (Mano *et al*., 2007) (Figure 7). This process converts gemfibrozil to a potent mechanism-based inhibitor of CYP2C8 (Ogilvie *et al*., 2006). CYP2C8 catalyses benzylic oxidation of the glucuronide, which leads to haem alkylation and inactivation of CYP2C8 (Baer *et al*., 2009; Jenkins *et al*., 2011). Inhibition of CYP2C8 by gemfibrozil 1-O-β-glucuronide was estimated to lead to k_{inact} value of 0.21 min^{-1} in human liver microsomes (HLM). K_I values were determined to be 20 and 52 µM using microsomal concentrations of 0.1 and 1.0 mg/ml, respectively (Ogilvie *et al*., 2006).

Table 10. Reported *in vitro* inhibitory effects of gemfibrozil and gemfibrozil 1-O-β-glucuronide on some enzymes and transporters.

Enzyme/ transporter	*Effects of gemfibrozil*		*Effects of gemfibrozil 1-O-β-glucuronide*	
CYP1A2	IC_{50} 99 µM	(Ogilvie *et al*., 2006)	NS at 300 µM	(Ogilvie *et al*., 2006)
	K_i 82 µM	(Wen *et al*., 2001)		
CYP2A6	NS at 250 µM	(Wen *et al*., 2001)	-	
CYP2B6	NS at 300 µM	(Ogilvie *et al*., 2006)	NS at 300 µM	(Ogilvie *et al*., 2006)
CYP2C8	IC_{50} 120 µM	(Ogilvie *et al*., 2006)	IC_{50} 4.07 µM	(Shitara *et al*., 2004)
	IC_{50} 28 µM	(Shitara *et al*., 2004)	IC_{50} decreased from 24 to 1.8 µM after a 30-min preincubation	(Ogilvie *et al*., 2006)
	K_i 55.4 µM	(Fujino *et al*., 2003)		
CYP2C9	IC_{50} 30 µM	(Ogilvie *et al*., 2006)	NS at 300 µM	(Ogilvie *et al*., 2006)
	K_i 5.8 µM	(Wen *et al*., 2001)		
	K_i 18.6 µM	(Fujino *et al*., 2003)		
CYP2C19	IC_{50} 100 µM	(Ogilvie *et al*., 2006)	NS at 300 µM	(Ogilvie *et al*., 2006)
	K_i 24 µM	(Wen *et al*., 2001)		
CYP2D6	NS at 250 µM	(Wen *et al*., 2001)	-	
CYP2E1	NS at 250 µM	(Wen *et al*., 2001)	-	
CYP3A4	NS at 250 µM	(Wen *et al*., 2001)	IC_{50} 243 µM	(Shitara *et al*., 2004)
	K_i 171 µM	(Fujino *et al*., 2003)	NS at 300 µM	(Ogilvie *et al*., 2006)
	IC_{50} 372 µM	(Shitara *et al*., 2004)		
	NS at 300 µM	(Ogilvie *et al*., 2006)		
UGT	IC_{50} 354 µM in HLM*	(Prueksaritanont *et al*., 2002)	IC_{50} 130 µM after a 25-min preincubation in HLM**	(Gan *et al*., 2010)
	IC_{50} 113 µM**	(Gan *et al*., 2010)		
UGT1A1	-		IC_{50} 69 µM after a 25-min preincubation	(Gan *et al*., 2010)
MRP2	IC_{50} > 250 µM	(Yamazaki *et al*., 2005)	NS at 100 µM	(Nakagomi-Hagihara *et al*., 2007b)
	NS at 100 µM	(Nakagomi-Hagihara *et al*., 2007b)		
NTCP	IC_{50} 23µM	(Ho *et al*., 2006)	-	
OAT3	IC_{50} 6.8 µM	(Nakagomi-Hagihara *et al*., 2007a)	19.7 µM	(Nakagomi-Hagihara *et al*., 2007a)
OATP1B1	IC_{50} 72.4 µM	(Shitara *et al*., 2004)	IC_{50} 24.3 µM	(Shitara *et al*., 2004)
	K_i 4 µM	(Schneck *et al*., 2004)	K_i 22.6 µM	(Hirano *et al*., 2006)
	K_i 12.5 µM	(Yamazaki *et al*., 2005)	K_i 15.7 µM	(Nakagomi-Hagihara *et al*., 2007b)
	K_i 25.2 µM	(Hirano *et al*., 2006)		
	IC_{50} 25 µM	(Ho *et al*., 2006)		
	K_i 31.7 µM	(Nakagomi-Hagihara *et al*., 2007b)		
	IC_{50} 63 µM (substrate-dependent inhibition)	(Noé *et al*., 2007)		
OATP1B3	NS at 100 µM	(Ho *et al*., 2006)	-	
	62% inhibition at 200 µM	(Noé *et al*., 2007)		
OATP2B1	IC_{50} 8µM	(Ho *et al*., 2006)	-	
	70% inhibition at 200 µM	(Noé *et al*., 2007)		
P-gp	IC_{50} > 250 µM	(Yamazaki *et al*., 2005)	-	

-, data not available; MRP2, multidrug resistance-associated protein 2; NS, no significant inhibition (< 50%); NTCP, sodium-dependent taurocholate cotransporting polypeptide; OAT3, organic anion transporter 3; OATP, organic anion-transporting polypeptide; P-gp, P-glycoprotein; *, inhibition of simvastatin glucuronidation in human liver microsomes (at least UGT1A1 and UGT1A3 involved); **, inhibition of repaglinide glucuronidation in human liver microsomes (at least UGT1A1 and UGT1A3 involved).

The pharmacokinetic interactions caused by gemfibrozil may be mediated by several mechanisms. Both parent gemfibrozil and gemfibrozil 1-O-β-glucuronide can interfere with drug metabolism and transport. Reported *in vitro* inhibitory effects of gemfibrozil and gemfibrozil 1-O-β-glucuronide on some enzymes and transporters are provided in Table 10. *In vitro*, the inhibitory effect of gemfibrozil on CYP2C9 is more potent than on CYP2C8. In addition to the *in vitro* inhibitory effects shown in Table 10, gemfibrozil has been reported to induce at least CYP2C8, CYP3A4 and UGT1A1 *in vitro* (Prueksaritanont *et al*., 2005).

In vivo gemfibrozil increases the AUC of a number of CYP2C8 substrate drugs such as repaglinide (AUC increased by 8.1-fold compared to control), cerivastatin (5.6-fold), montelukast (4.5-fold), pioglitazone (3.2-3.4-fold), rosiglitazone (2.3-fold), and loperamide (2.2-fold) (Niemi *et al*., 2003a; Deng *et al*., 2005; Jaakkola *et al*., 2005; Niemi *et al*., 2005; Niemi *et al*., 2006; Karonen *et al*., 2010), but it did not increase the AUC of CYP2C9 substrate warfarin (Lilja *et al*., 2005). The mechanism-based inhibition of CYP2C8 by gemfibrozil 1-O-β-glucuronide can probably explain the differences in *in vitro* and *in vivo* potencies of CYP2C8 and CYP2C9 inhibition by gemfibrozil (Ogilvie *et al*., 2006).

The AUC of other drugs has also increased with concomitant gemfibrozil administration *in vivo*. E.g., atorvastatin AUC increased by 1.2-1.4-fold, and moderate increases were seen also in the AUCs of atorvastatin metabolites (Backman *et al*., 2005; Whitfield *et al*., 2011). Similarly, the AUC of simvastatin increased by 1.4-fold and its acid metabolite by 2.5-fold; the AUC of active lovastatin acid increased by 2.8-fold and that of pravastatin by 2.0-fold with gemfibrozil coadministration (Backman *et al*., 2000; Kyrklund *et al*., 2001; Kyrklund *et al*., 2003).

10. Repaglinide

Repaglinide is an oral insulin secretagogue of the meglitinide class. It was developed to be used in the treatment of type II diabetes, either as monotherapy or combined with other oral antihyperglycaemic agents such as metformin, in the 1990's (Guay, 1998). Repaglinide was approved for clinical use in the United States in 1997 and in Europe in 2001. Repaglinide closes the adenosine triphosphate (ATP)-dependent potassium channels in the pancreatic β-cells, which leads to the depolarisation of the cell and the subsequent activation of voltage-dependent calcium channels. Thereafter calcium can enter the cells and initiate insulin secretion (Gromada *et al.*, 1995). The basic characteristics of repaglinide are shown in Table 11.

Table 11. Characteristics of repaglinide (Guay, 1998; van Heiningen *et al.*, 1999; Plum *et al.*, 2000; Dornhorst, 2001; Hatorp, 2002; Powers and D'Alessio, 2010).

Chemical structure	S (+) 2-ethoxy-4- [2-oxo-2-[(α-isobutyl-2-piperidinobenzyl) amino] ethyl]-benzoic acid, $C_{27}H_{36}N_2O_4$
Molecular weight	452.6
Usual daily dosage	0.5 – 4 mg 4 times daily (before meals)
Oral bioavailability (F)	~60%
$t_{1/2}$	1-1.5 h
Plasma protein binding	98.5% (mainly to albumin)
Volume of distribution (V_d)	31 l
Metabolism	CYP2C8 and CYP3A4
Route of excretion	Metabolites: biliary (90%), urinary (8%); as unchanged (2%)

10.1. Repaglinide metabolism, interactions and pharmacogenomics

Repaglinide is metabolised mainly by CYP2C8 and CYP3A4 to inactive metabolites (Bidstrup *et al.*, 2003; Kajosaari *et al.*, 2005a). The metabolites include M1 (an aromatic amine; ~4% of dose), M2 (an oxidised dicarboxylic acid; ~66% of dose) and M4 (formed via hydroxylation of the piperidine ring; ~1% of dose). Approximately 87% of the repaglinide dose is biotransformed through oxidative metabolism (Bidstrup, 2006). The main metabolic routes of repaglinide *in vitro* are shown in Figure 8. The formation of M2 has been proposed to be a multi-step reaction mediated by CYP3A4 and an unknown cytosolic enzyme (Gan *et al.*, 2010). Also low quantities of other metabolites such as glucuronides formed by UGT1A1 have been detected *in vitro* (Gan *et al.*, 2010). CYP2C8 is considered as the most important enzyme in repaglinide metabolism; the fraction metabolised by CYP2C8 ($f_{m,CYP2C8}$) has been estimated to be between 0.49 and 0.61 (Hinton *et al.*, 2008; Baer *et al.*, 2009).

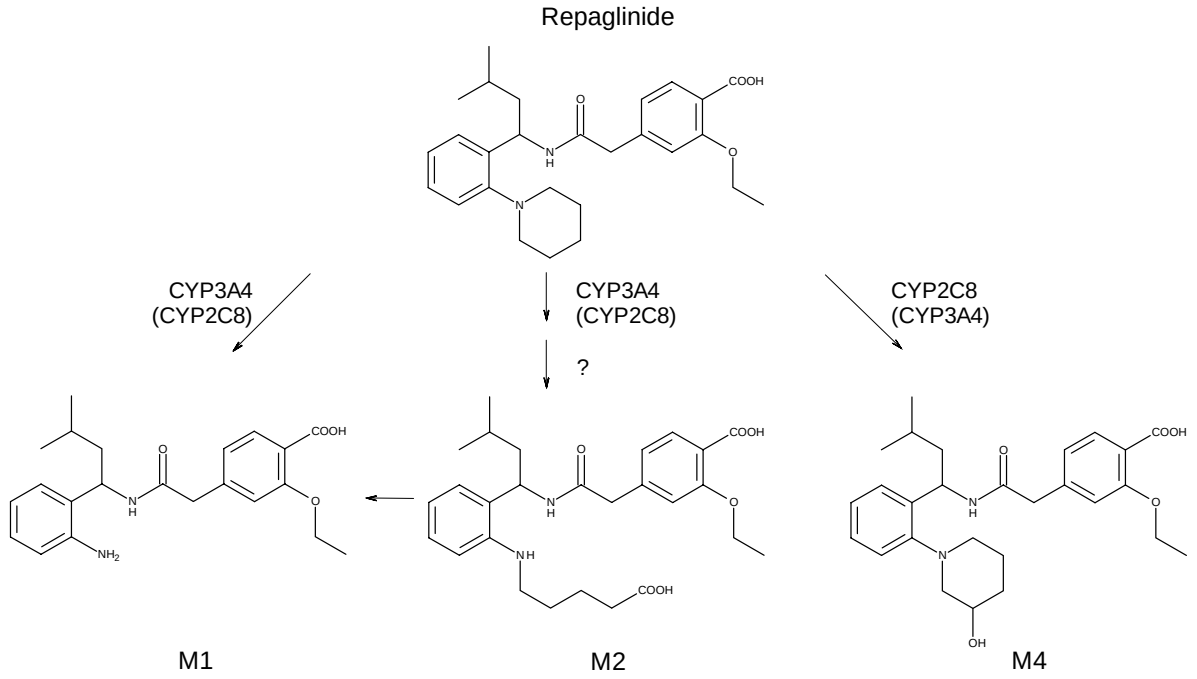

Figure 8. Chemical structures of repaglinide, its M1, M2 and M4 metabolites and principal enzymes catalysing the reactions *in vitro* (van Heiningen *et al*., 1999; Bidstrup *et al*., 2003; Kajosaari *et al*., 2005a; Kajosaari *et al*., 2005b; Gan *et al*., 2010).

CYP2C8 inhibitors gemfibrozil and trimethoprim inhibit repaglinide metabolism both *in vitro* and *in vivo*. In *in vitro* studies, the IC_{50} values of gemfibrozil and trimethoprim have been estimated to be 111 µM and 129 µM, respectively (Niemi *et al*., 2004; Kajosaari *et al*., 2005a). *In vivo*, gemfibrozil and trimethoprim have increased the AUC of repaglinide by 8.1- and 1.6-fold, respectively (Niemi *et al*., 2003b; Niemi *et al*., 2004). The formation of CYP2C8-dependent repaglinide metabolite M4 decreases (Tornio *et al*., 2008a) and that of CYP3A4-dependent metabolite M1 increases (Niemi *et al*., 2003b) with gemfibrozil administration. The *CYP2C8* genotype also affects repaglinide pharmacokinetics. The plasma concentrations of repaglinide are smaller in carriers of the *CYP2C8*3* allele than in noncarriers. It has been shown that repaglinide AUC was 45% lower in subjects with the *CYP2C8*1/CYP2C8*3* genotype compared to the *CYP2C8*1/CYP2C8*1* genotype (Niemi *et al*., 2003c). However, this finding has not been confirmed in two other studies (Bidstrup *et al*., 2006; Tomalik-Scharte *et al*., 2011).

CYP3A4 inhibitors also inhibit repaglinide metabolism, although the increases in repaglinide AUC with them have been smaller than with CYP2C8 inhibitors. In clinical drug-drug interaction studies clarithromycin and grapefruit juice, which are mechanism-based inhibitors of CYP3A4, increased the AUC of repaglinide only by 1.4- and 1.13-fold, respectively (Niemi *et al*., 2001; Bidstrup *et al*., 2006). Ketoconazole, itraconazole and telithromycin, reversible inhibitors of CYP3A4, increased the repaglinide AUC by 1.15-, 1.4- and 1.8-fold, respectively (Hatorp *et al*., 2003; Niemi *et al*., 2003b; Kajosaari *et al*., 2006). The formation of CYP3A4-dependent metabolite M1 decreased markedly compared to control in these studies (Kajosaari *et al*., 2006; Niemi *et al*., 2006). Interestingly, the concentrations of M2,

also proposed to be formed by CYP3A4, did not decrease with concomitant treatment of CYP3A4 inhibitor telithromycin (Kajosaari *et al*., 2006). When combining itraconazole with gemfibrozil to achieve an inhibition of both CYP3A4 and CYP2C8, a 19.4-fold increase in the repaglinide AUC was seen. The concentrations of M1 were decreased by the combination (Niemi *et al*., 2003b).

Rifampicin, a classic enzyme inducer, has been shown to both induce and competitively inhibit repaglinide metabolism by CYP3A4 and CYP2C8 (Niemi *et al*., 2000; Kajosaari *et al*., 2005a) and to inhibit OATP1B1 (Benet, 2009). As competitive inhibition starts and stops quickly according to the changes in the inhibitor concentrations, and induction is a relatively slow process; the *in vivo* effects of rifampicin on repaglinide pharmacokinetics depend on the time interval with the administration of the drugs. When repaglinide was taken simultaneously with the last dose of a 7-day course of rifampicin, the AUC of repaglinide was decreased by 50%. When rifampicin was discontinued earlier, already 24 h before repaglinide intake, repaglinide AUC was decreased even more, by 80%. This was proposed to be due to the rapid disappearance of the inhibitory effect of rifampicin, whilst induction was still present (Bidstrup *et al*., 2004).

The hepatic transporter organic anion-transporting polypeptide 1B1 (OATP1B1), encoded by the solute carrier organic anion transporter 1B1 (*SLCO1B1*) gene in chromosome 12p12, participates in the uptake of repaglinide from blood to hepatocytes (Niemi *et al*., 2011). Therefore, changes in the OATP1B1 activity, e.g., due to concomitant drugs or genetic factors, may affect repaglinide pharmacokinetics. Gemfibrozil and gemfibrozil 1-O-β-glucuronide both inhibit OATP1B1 (Shitara *et al*., 2004). Polymorphisms in the *SLCO1B1* gene have also been shown to affect repaglinide pharmacokinetics (Kalliokoski *et al*., 2008a). *SLCO1B1*5* and **15* have been associated with decreased transport activity, whilst *SLCO1B1*1B* has been associated with increased activity. These changes in transport activity have been associated with increased and decreased repaglinide plasma concentrations, respectively. Decreased OATP1B1 activity increases also the concentrations of M2 and M4 (Kalliokoski *et al.*, 2008a; Kalliokoski *et al.*, 2008b; Kalliokoski and Niemi, 2009).

In addition, OATP1B1 inhibitor cyclosporine, which is also a CYP3A4 inhibitor, has increased the AUC of repaglinide 2.4-fold (Kajosaari *et al*., 2005b; Backman *et al*., 2006). Concurrently, the urinary excretion of unchanged repaglinide and metabolites M2 and M4 increased by 2.7-, 7.5- and 5.0-fold, respectively. The urinary excretion of CYP3A4-dependent metabolite M1 was not affected, although the M1/repaglinide ratio decreased by 62% (Kajosaari *et al*., 2005b). The potential of cyclosporine to inhibit P-glycoprotein is not considered to affect repaglinide disposition, as repaglinide has been shown not to be a P-glycoprotein substrate (Kajosaari *et al*., 2005b).

Currently, there is no clear evidence that repaglinide would interfere with the metabolism or transport of other drugs (Scheen, 2007). There are some reports suggesting that repaglinide might inhibit the OATP- or organic cation transporter (OCT) -mediated transport of some compounds (Bachmakov *et al*., 2008).

AIMS OF THE STUDY

The aim of this study was to characterise the time- and dose-dependency of the effect of gemfibrozil on the pharmacokinetics of repaglinide in order to elucidate the mechanism-based inhibitory effect of gemfibrozil on CYP2C8 in humans. The specific aims of the study were:

1. To study the onset of mechanism-based inhibition of CYP2C8 by gemfibrozil in humans.

2. To study the persistence of CYP2C8 inhibition caused by gemfibrozil in humans.

3. To study the dose relationship of CYP2C8 inhibition caused by gemfibrozil in humans.

4. To obtain new data concerning CYP2C8 turnover and the CYP2C8 inactivation potency of gemfibrozil, for use when assessing CYP2C8 related drug interaction potential of new chemical entities.

MATERIALS AND METHODS

This work was carried out as four *in vivo* clinical drug-drug interaction studies at the Department of Clinical Pharmacology, University of Helsinki. The study protocols were approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District, by the Helsinki University Central Hospital (HUCH) and by the Finnish National Agency for Medicines / Finnish Medicines Agency.

1. Subjects

The subjects in the studies were healthy volunteers. Before entering the studies, they had been given both oral and written information and they had given a written informed consent.

The number of subjects in the studies was estimated to be sufficient to detect a 30% difference between the phases in the $AUC_{0-\infty}$ of repaglinide with a power of > 80% (alpha-level 5%). The sample size calculation for all studies ended up to 9.3 subjects / group. As the studies were designed as cross-over studies (all subjects completing all phases), a sample size of 10 was chosen for studies I, II and III. For balancing reasons (four phases), and due to the long clinical phase (possibility of drop-outs), altogether 12 subjects entered study IV (Table 12).

One subject participated in both studies I and II. Four subjects participated in both studies III and IV. One subject was excluded from the analyses due to suspected non-compliance of the study protocol (II) and two subjects discontinued their participation due to reasons not related to the study (IV). The analyses were based on the data of 39 (27 male and 12 female) subjects (Table 12).

Table 12. Characteristics of the subjects included in the analyses.

Study	*Subjects n (male/female)*	*Age (y)*	*Weight (kg)*	*Body mass index (kg/m^2)*
I	10 (5/5)	26 ± 4	72 ± 16	24 ± 4
II	9 (7/2)	22 ± 2	77 ± 9	24 ± 2
III	10 (9/1)	23 ± 2	73 ± 10	23 ± 2
IV	10 (6/4)	24 ± 3	75 ± 9	25 ± 2

Age, weight and body mass index are presented as mean ± standard deviation (SD).

The subjects were ascertained to be healthy by medical history, clinical examination and routine laboratory tests. None of the subjects was using continuous medication or oral contraception, or was a smoker. The female subjects gave a negative pregnancy test before entering and during the studies. Participation in other studies and blood donation were prohibited 2 months before and during the studies. The use of alcohol, grapefruit and any medications was prohibited 1 week, and rigorous physical exercise 1 day prior to each study day.

2. Study design

Studies I and II were designed to evaluate the time effects and studies III and IV the dose effects of gemfibrozil on CYP2C8 inhibition. All four studies were of randomised, balanced and cross-over design. Studies I and II assessing the time effect were open-label, whilst studies III and IV assessing the dose effect were double-blinded. In all studies, a small 0.25 mg dose of repaglinide was used as an *in vivo* probe for CYP2C8 activity. The studies differed from each other by the gemfibrozil pre-treatment. Wash-out periods of 2-3 weeks between the phases were held in order to prevent carry-over effect. Details of the study designs are given in Table 13.

Table 13. Study designs.

Study	*Phases (n)*	*Blinding*	*Control phase*	*Gemfibrozil pretreatment dosing*	*Pretreatment timing*
I	5	Open	Repaglinide alone	Gemfibrozil single dose 600 mg	0, 1, 3 or 6 h before repaglinide
II	5	Open	Repaglinide alone	Gemfibrozil 600 mg twice daily for 3 days	Last pre-treatment dose 1, 24, 48 or 96 h before repaglinide
III	5	Double-blinded	Repaglinide with placebo	Gemfibrozil single dose 30, 100, 300 or 900 mg	Pretreatment 1 h before repaglinide
IV	4	Double-blinded	Repaglinide with placebo	Gemfibrozil 30, 100 or 600 mg twice daily for 5 days	Last pre-treatment dose 1 h before repaglinide

2.1. Study drugs

For safety reasons, a small 0.25 mg dose of oral blood glucose lowering drug repaglinide was used as an *in vivo* probe for CYP2C8 activity. NovoNorm 0.5 mg tablets (Novo Nordisk, Bagsværd, Denmark) provided by the HUCH pharmacy were halved and weighed by the same investigator for all studies. For halving the tablets, in each study, 10 tablets were weighed together, and by dividing the mass by 20, a target mass for the tablet halves was obtained. Subsequent tablet halves with a maximum of one percent (1%) deviation from this target mass were accepted for use in the study. After halving the tablets for each study, the tablet halves were organised in order of increasing weight, and every subject received tablet halves of approximately the same weight.

For gemfibrozil pre-treatment in studies I and II Lopid 600 mg tablets (Gödecke, Freiburg, Germany) were provided by the HUCH pharmacy. For studies III and IV, gemfibrozil and placebo capsules were prepared using methods described in the European Pharmacopoeia (Ph. Eur.) by the HUCH Pharmacy. The gemfibrozil content of the capsules was measured using the liquid chromatography–tandem mass spectrometry system. The randomisation for all

studies was done by the HUCH Pharmacy. The blinding codes for studies III and IV were opened after the clinical phase of the studies.

Gemfibrozil/placebo capsules were supplied to the subjects in advance in studies I, II and IV. Gemfibrozil/placebo capsules in study III and repaglinide tablets in all studies were administered to the subjects in the study premises.

2.2. Study conduct

The study days (n = 46) started at 7:30 in the morning with insertion of intravenous forearm cannules to the subjects. During the study days, the subjects were under direct medical supervision. Blood sampling and drug administration started at 8:00.

The study drugs were administered orally after an overnight fastening with 100 ml (with pre-treatment) and 150 ml (with repaglinide) water. The subjects remained seated until 12:00 in order to keep the conditions for drug absorption as identical as possible.

Timed blood samples (5 or 10 ml each) were drawn from a cannulated forearm vein 60, 30 and 5 min before and at 15, 30, 45, 60, 80 and 100 min, and 2, 2.5, 3, 4, 5, 7 and 9 h after the administration of repaglinide into ethylenediaminetetraacetic acid (EDTA) containing tubes. In study II, blood samples for the determination of gemfibrozil and gemfibrozil 1-O-β glucuronide concentrations were also taken during the 96 h timing interval (25, 49 and 73 h before repaglinide intake) and during the 48 h timing interval (25 h before repaglinide intake). Plasma was separated within 30 min and stored at -70ºC until analysis.

From samples taken 5 min before and at 15, 30, 45, 60, 80 and 100 min, and 2, 2.5, 3, 4, 5, 7 and 9 h after the administration of repaglinide blood glucose concentrations were measured immediately after sampling. The measurement was made by a Precision G Blood Glucose Testing System (Medisense, Bedford, MA) in studies I and II, and by a Precision Exceed device (Abbott Diabetes Care Ltd, Witney Oxon, UK) in studies III and IV.

The last samples for pharmacokinetic analyses were taken at 18:00. If the subjects' blood glucose levels were equal to or exceeded the predefined safety level (4.0 mmol/l), the study day was regarded as ended and the subjects were allowed to leave the study premises. In other cases, additional carbohydrates were provided and blood glucose levels were monitored until reaching the safe minimum level.

Food intake was identical during all study days to provide accuracy for pharmacodynamic data (blood glucose levels). The meals comprised a standardised light breakfast 15 min after repaglinide administration (eaten over duration of 10 min), carbohydrate-rich snacks after 1 and 2 h (eaten over duration of 5 min), a warm meal after 3 h, and snacks after 7 and 9 h.

Additional carbohydrates, glucose solution for intravenous use and glucagon for intramuscular use, as well as adrenaline and equipment for resuscitation were available.

2.3. Genotyping

For genotyping, a 12-20 ml EDTA blood sample was drawn from each subject and stored at -20°C. Genomic deoxyribonucleic acid (DNA) was extracted with standard methods (Qiaamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). The subjects were genotyped for the *CYP2C8*1* (wild type), *CYP2C8*3* (c.416G>A and c.1196A>G) and *CYP2C8*4* (c.792C>G) alleles and the *SLCO1B1*1A* (wild type) and *SLCO1B1* c.388A>G and c.521T>C single nucleotide polymorphisms (SNP), defining the *SLCO1B1*1B* (GT), **5* (AC), and **15* (GC) haplotypes (Kalliokoski and Niemi, 2009), with TaqMan® genotyping assays on an Applied Biosystems 7300 Real-Time PCR system (Pasanen *et al*., 2006). The *CYP2C8* and *SLCO1B1* genotypes of the subjects included in the analyses are presented in Table 14.

Table 14. Numbers of different *CYP2C8* and *SLCO1B1* genotype carriers in the studies.

Study	*CYP2C8 genotype (n of subjects)*	*SLCO1B1 genotype (n of subjects)*
I	**1/*1* (10)	**1A/*1A* (3) **1A/*1B* (2) **1A/*15* (3) **1B/*15* (1) **15/*15* (1)
II	**1/*1* (6) **1/*3* (2) **1/*4* (1)	**1A/*1A* (2) **1A/*1B* (2) **1A/*15* (2) **1B/*15* (1) **15/*15* (2)
III	**1/*1* (8) **1/*3* (1) **3/*3* (1)	**1A/*1A* (3) **1A/*1B* (3) **1A/*15* (3) **1B/*15* (1)
IV	**1/*1* (8) **1/*3* (1) **3/*3* (1)	**1A/*1B* (3) **1A/*15* (4) **1B/*1B* (1) **1B/*15* (2)

3. Determination of drug concentrations

3.1. Repaglinide and its metabolites

Concentrations of repaglinide and its metabolites M1, M2, and M4 were measured in plasma samples by use of an API 3000 liquid chromatography–tandem mass spectrometry system (Sciex Division of MDS, Toronto, Ontario, Canada) (Tornio *et al*., 2008a). The limit of quantification for repaglinide was 0.01 ng/ml in all studies. In all studies except II, the limit of quantification for repaglinide M1 and M2 was 0.02 ng/ml. Because an authentic metabolite standard for M4 was not available, M4 concentrations are given in arbitrary units (units per millilitre) relative to the ratio of the peak height of M4 to that of the internal standard in the chromatogram. The limit of quantification for M4 was based on a signal-to-noise ratio of

more than 10:1. This approach was used also for M1 and M2 in study II. Gemfibrozil or its glucuronide did not interfere with the assays.

3.2. Gemfibrozil and gemfibrozil 1-O-β-glucuronide

The plasma concentrations of gemfibrozil and gemfibrozil 1-O-β-glucuronide were determined by the use of the Applied Biosystems API 2000 Q Trap liquid chromatography–tandem mass spectrometry system (Sciex Division of MDS, Toronto, Ontario, Canada) using a modification of a previous method (Roadcap *et al*., 2003). Gemfibrozil-d6 and gemfibrozil 1-O-β-glucuronide-d6 served as internal standards. The limits of quantification for gemfibrozil and gemfibrozil 1-O-β-glucuronide were 0.0025 mg/l in all studies.

4. Pharmacokinetic calculations

The pharmacokinetics of repaglinide and its metabolites M1, M2, and M4 were characterised by the peak concentration (C_{max}), $AUC_{0\text{-}9h}$ and $AUC_{0\text{-}\infty}$ ($AUC_{0\text{-}3h}$ for M4) and elimination half-life ($t_{½}$). The terminal log-linear part of each concentration-time curve was identified visually. The elimination rate constant (k_e) was determined by linear regression analysis of the log-linear part of the plasma concentration-time curve. The $t_{½}$ was calculated by the equation $t_{½} = \ln 2/k_e$. The AUC values were calculated by use of the linear trapezoidal rule for the rising phase of the plasma repaglinide concentration-time curve and the log-linear trapezoidal rule for the descending phase, with extrapolation to infinity, when appropriate, by dividing the last measured concentration by k_e. In addition, in study II, the oral clearance (CL/F) of repaglinide was calculated by dividing its dose (0.25 mg) with its $AUC_{0\text{-}\infty}$.

The pharmacokinetics of gemfibrozil and gemfibrozil 1-O-β-glucuronide were characterised by concentration at different times (C_t), C_{max}, time to C_{max} (t_{max}), $t_{½}$ and different $AUC_{0\text{-}t}$. The elimination rate constant (k_e) was determined by linear regression analysis of the log-linear part of the plasma concentration-time curve. In study IV, the C_{12} and $AUC_{0\text{-}12h}$ were extrapolated from C_{10} and $AUC_{0\text{-}10h}$ using the k_e. In addition, the average concentrations ($C_{avg,0\text{-}t}$) were calculated by dividing the $AUC_{0\text{-}t}$ by time (t). In study II, $C_{avg,0\text{-}9h}$; in study III, $C_{avg,0\text{-}10h}$ and in study IV, $C_{avg,0\text{-}12h}$ were calculated.

The pharmacokinetics were calculated by noncompartmental analysis using WinNonlin, version 5.2 (Pharsight Inc., Mountain View, CA) for study II, and MK-Model, version 5.0 (Biosoft, Cambridge, UK) for studies I, III and IV.

5. Pharmacodynamic calculations

The pharmacodynamics of repaglinide was measured by changes in blood glucose levels. They were characterised by baseline (i.e. before administration of repaglinide), minimum and mean blood glucose concentration (from 0 to 3 and 9 h). The mean blood glucose concentrations were calculated by dividing the area under the blood glucose concentration-time curve by the corresponding time interval.

6. Statistical analyses

Pharmacokinetic and pharmacodynamic variables between the phases were tested using a paired t-test, or in the case of t_{max}, by the Wilcoxon signed-rank test. Before statistical testing, pharmacokinetic variables in studies I and IV were log-transformed, when appropriate. To avoid false negative conclusions and because the direction of the interaction has been documented previously, no Bonferroni correction for multiple comparisons was applied. Differences were therefore considered statistically significant at $P < 0.05$ in all studies.

In studies III (single dose) and IV (multiple dose) the dose-proportionality of gemfibrozil pharmacokinetics was estimated by regression analysis with the power-model approach using a logarithmically transformed form of the equation:

$$AUC = e^{\alpha} \cdot dose^{\beta}$$

after logarithmic transformation of the gemfibrozil and its glucuronide $AUC_{0\text{-}\infty}$ data (study III) or $AUC_{0\text{-}12h}$ data (study IV). In this approach a statistically significant deviation of the term β from unity indicated dose non-linearity.

Studies I and II were analysed with SPSS for Windows, version 16.0.1 (SPSS Inc, Chicago, IL) and studies III and IV with PASW for Windows, version 17.0 (SPSS Inc, Chicago, IL).

7. Application of physiological concepts and models to *in vivo* interaction data using regression analyses

7.1. Estimation of CYP2C8 turnover half-life

Using the extent of the interaction (fractional decrement in oral clearance (FDCL) from repaglinide $AUC_{inhibited}/AUC_{control}$ data) at different time points after stopping gemfibrozil administration, the first-order degradation rate constant of CYP2C8 was estimated in study II. The interaction data from time points 24, 48 and 96 h obtained in the study were used for the analysis. The time point for the control phase representing full recovery of the enzyme activity was set to 10,000 h. For the estimation, the oral clearance (CL/F) of repaglinide was described with the following equation:

$$CL/F(t) = CL/F_{recovered} - FDCL_{24} \cdot e^{-k_{deg} \cdot (t-24h)} \cdot CL/F_{recovered}$$

where $CL/F_{recovered}$ is the CL/F of repaglinide when CYP2C8 activity is fully recovered, $FDCL_{24}$ is the fractional decrement in the CL/F of repaglinide when repaglinide is administered at 24 h after the last dose of gemfibrozil, k_{deg} is the first-order degradation rate constant of CYP2C8, and t is the time after the last dose of gemfibrozil. This equation was fitted to the data from the study by non-linear regression analysis, with the estimation of k_{deg}. From the k obtained, the degradation half-life of CYP2C8 was calculated using the equation $t_{½} = \ln 2/k_{deg}$.

7.2. Pharmacokinetic modelling

Pharmacokinetic modelling was done to evaluate the repaglinide fraction metabolised by CYP2C8 ($f_{mCYP2C8}$), intrahepatic inhibitor (gemfibrozil / gemfibrozil 1-O-β-glucuronide) concentrations and to confirm the mechanism-based inhibition of CYP2C8 to be the main mechanism for repaglinide-gemfibrozil interaction. Also, the contribution of possible OATP1B1 inhibition to this interaction was examined by mathematical modelling.

Several static enzyme and transporter inhibition models were applied to the relationship between the plasma concentrations of gemfibrozil or its 1-O-β-glucuronide and the increase in the AUC of repaglinide in the studies III and IV. Previously published IVIVE equations were fitted to the repaglinide AUC fold increase data (Mayhew *et al*., 2000). *In vitro* constants for CYP2C8 (k_{inact} 0.21 min^{-1}, K_I 20 µM) and OATP1B1 inhibition (IC_{50} 24.3 and 72.4 µM for gemfibrozil and its glucuronide, respectively) published in literature (Shitara *et al*., 2004; Ogilvie *et al*., 2006; Hinton *et al*., 2008) were used in the calculations. Also, the degradation rate constant for CYP2C8 (k_{deg}) obtained from study II was used in the models including a mechanism-based inhibition component. The ratio of unbound hepatocyte concentration vs. total plasma concentration ($C_{h,u}/C_{p,tot}$) of gemfibrozil or its glucuronide, repaglinide $f_{m,CYP2C8}$ and fraction transported by OATP1B1 ($f_{t,OATP1B1}$) were left as unknown parameters to be predicted with non-linear regression analysis. The fit of the models (coefficient of determination, r^2) and the sanity of the estimated parameters were used for assessing the possibility of the tested interaction mechanism to explain the observed repaglinide AUC fold increase. In some cases, predefined, meaningful constraints for the parameters to be estimated were used in the regression analyses (e.g., $0 \leq f_{m,CYP2C8} \leq 1$). The equations tested are presented in Table 15.

Table 15. Equations used in the non-linear regression analyses concerning the dose-dependency of gemfibrozil-repaglinide interaction in studies III and IV.

Inhibitor and inhibition type	*Equation (AUC fold increase =)*
Gemfibrozil 1-O-β-glucuronide	
mechanism-based CYP2C8	$1/[(f_{m,CYP2C8}/(1+k_{inact}/K_I \cdot [I]_h/k_{deg}))+1-f_{m,CYP2C8}]$
competitive OATP1B1	$1/[(f_{t,OATP1B1}/(1+[I]_h/K_i))+1-f_{t,OATP1B1}]$
mechanism-based CYP2C8 + competitive OATP1B1	$(1/[(f_{m,CYP2C8}/(1+k_{inact}/K_I \cdot [I]_h/k_{deg}))+1-f_{m,CYP2C8}]) \cdot (1/[(f_{t,OATP1B1}/(1+[I]_{p,u}/K_i))+1-f_{t,OATP1B1}])$
Gemfibrozil	
competitive CYP2C8	$1/[(f_{m,CYP2C8}/(1+[I]_h/K_i))+1-f_{m,CYP2C8}]$
competitive OATP1B1	$1/[(f_{t,OATP1B1}/(1+[I]_h/K_i))+1-f_{t,OATP1B1}]$

$f_{m,CYP2C8}$, fraction metabolised by CYP2C8; $f_{t,OATP1B1}$, fraction transported by OATP1B1; $[I]_h$, inhibitor concentration in the liver; $[I]_{p,u}$, plasma unbound inhibitor concentration

In the equations, both average and peak plasma concentrations gemfibrozil and gemfibrozil 1-O-β-glucuronide were used separately. As the potential of competitive inhibition of CYP2C8 by gemfibrozil 1-O-β-glucuronide has been shown to be very weak compared to mechanism-based inhibition (Shitara *et al*., 2004), a model testing this inhibition type was not applied.

From equations including only one inhibition mechanism, the model providing the best fit (mechanism-based inhibition model) was also used for individual data. This was done both in studies III and IV. From this analysis, individual $f_{m,CYP2C8}$ and $C_{h,u}/C_{p,tot}$ values were obtained.

To examine, whether adding an OATP1B1 inhibition component to the mechanism-based CYP2C8 inhibition model would increase the fit of the models (r^2), a combined reversible OATP1B1 inhibition and time-dependent CYP2C8 inhibition model was also applied. This was done both in studies III and IV. The following equation was used:

$$\frac{AUC_i}{AUC_c} = \frac{1}{\frac{f_{m,CYP2C8}}{1+\left(\frac{k_{inact}\cdot[I]_h}{k_{deg}\cdot K_I}\right)} + \left(1 - f_{m,CYP2C8}\right)} \cdot \frac{1}{\frac{f_{t,OATP1B1}}{1+\left(\frac{[I]_{p,u}}{K_i}\right)} + \left(1 - f_{t,OATP1B1}\right)}$$

From this analysis, estimates for $f_{m,CYP2C8}$, $f_{t,OATP1B1}$ and $C_{h,u}/C_{p,tot}$ were obtained.

7.3. Estimation of remaining CYP2C8 activity and *in vivo* AUC ratios of CYP2C8 substrates with different gemfibrozil doses and different $f_{m,CYP2C8}$

Using the gemfibrozil dose-proportionality data (α, β) and the scaling factor for intrahepatic gemfibrozil 1-O-β-glucuronide ($C_{h,u}/C_{p,tot}$) obtained in study IV (multiple dosing), k_{deg} obtained from study II and previously published inhibition constants (k_{inact} and K_I), an estimation of the *in vivo* AUC ratios ($AUC_{inhibited}/AUC_{control}$) for substrate drugs of different fractions metabolised by CYP2C8 when treated with different gemfibrozil doses twice daily was done. The following equation with $f_{m,CYP2C8}$ values of 99%, 95%, 90%, 80%, 70% and 50% was used:

$$\frac{AUC_i}{AUC_c} = \frac{1}{\frac{f_{m,CYP2C8}}{1+\left(\frac{k_{inact}}{k_{deg}\cdot K_I}\cdot\frac{e^{\alpha}\cdot dose^{\beta}\cdot C_{h,u}/C_{p,tot}}{t}\right)} + \left(1 - f_{m,CYP2C8}\right)}$$

where t is the time of dosing interval of gemfibrozil (12 h).

In addition, estimates of the remaining CYP2C8 activity with different doses of gemfibrozil twice daily were made using the equation:

$$CYP2C8_{remaining}\ (\%) = \frac{100}{\frac{AUC_{inhibited}}{AUC_{control}}}$$

where the $AUC_{inhibited}/AUC_{control}$ values were taken from estimates with $f_{m,CYP2C8}$ of 0.99.

RESULTS

In this section, the main results of studies I-IV are presented. All pharmacokinetic data are presented here as fold changes of geometric means, although the original publications for studies II and III present fold changes of arithmetic means of the variables.

1. Onset of CYP2C8 inhibition (I)

CYP2C8 enzyme inhibition *in vivo*, reflected as changes in repaglinide metabolism in healthy volunteers, started rapidly after oral gemfibrozil administration. The changes in the concentrations of repaglinide and its metabolites were greatest when repaglinide was taken 1 or 3 h after gemfibrozil.

The geometric mean $AUC_{0-\infty}$ of repaglinide was 5.0-, 6.3-, 6.6- and 5.4-fold compared to control (i.e., no gemfibrozil) when gemfibrozil was taken simultaneously with and 1, 3 and 6 h before repaglinide, respectively (Figure 9; $P < 0.001$). The C_{max} of repaglinide increased by 1.4-, 2.1-, 2.1- and 2.0-fold compared to control, respectively ($P < 0.05$).

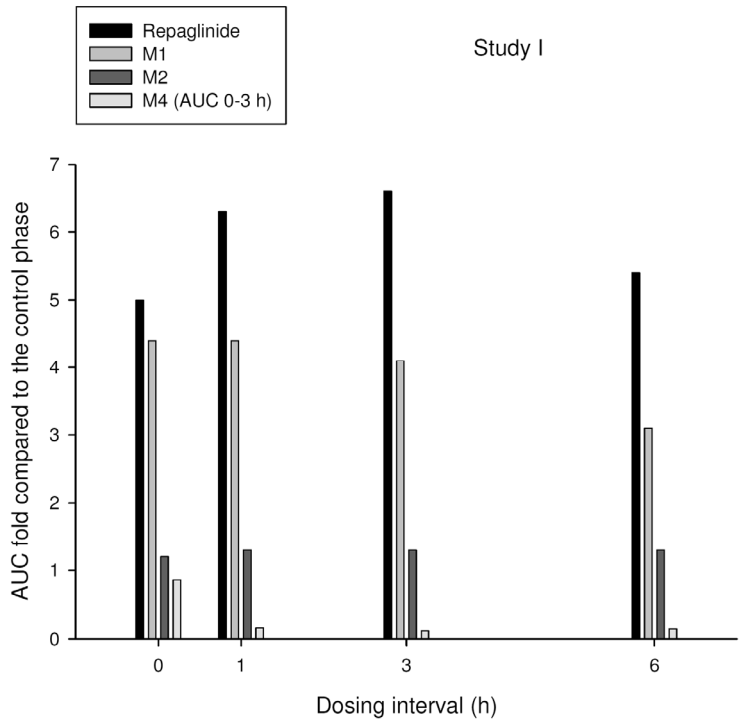

Figure 9. Fold changes in the AUC of repaglinide and its metabolites compared to the control phase, when a single 600 mg dose of gemfibrozil was taken simultaneously or 1, 3 or 6 h before repaglinide intake. For repaglinide M4, AUC_{0-3h} data is presented. For all other compounds, $AUC_{0-\infty}$ data is presented.

The formation of CYP3A4-dependent repaglinide metabolite M1 increased in the phases with gemfibrozil pretreatment. The $AUC_{0-\infty}$ of repaglinide M1 was 4.4-, 4.4-, 4.1- and 3.1-fold compared to the control phase, respectively (Figure 9; $P < 0.001$). The $AUC_{0-\infty}$ of repaglinide M2 was 1.2-, 1.3-, 1.3- and 1.3-fold, respectively (Figure 9; $P < 0.05$ in the 3 and 6 h dosing intervals). The formation of the CYP2C8-dependent repaglinide metabolite M4 was significantly impaired by gemfibrozil pretreatment. The AUC_{0-3h} of M4 was 85%, 16%, 12% and 15% of control when gemfibrozil was taken simultaneously with and 1, 3 and 6 h before repaglinide, respectively (Figure 9; $P < 0.001$ in the 1, 3 and 6 h dosing intervals). The M4/repaglinide AUC_{0-3h} ratio was 31%, 4%, 3% and 4% of control in the 0, 1, 3 and 6 h interval phases, respectively ($P < 0.001$ in the 1, 3 and 6 h dosing intervals).

2. Duration/persistence of CYP2C8 inhibition (II)

CYP2C8 inhibition was shown to recover slowly in humans. A practically complete recovery of the enzymatic activity to the control level, reflected by repaglinide metabolism, was detected 96 h after stopping twice daily gemfibrozil administration.

The geometric mean $AUC_{0-\infty}$ of repaglinide was 8.2-, 3.0-, 1.4- and 1.0-fold compared to control (i.e., no gemfibrozil) when the last dose of a 3-day gemfibrozil pretreatment with 600 mg twice daily was taken 1, 24, 48 and 96 h before repaglinide, respectively (Figure 10; $P < 0.001$ in the 1, 24 and 48 h phases). The C_{max} of repaglinide was 2.7-, 1.9-, 1.2- and 1.1-fold compared to control, respectively ($P < 0.001$ in the 1 and 24 h phases).

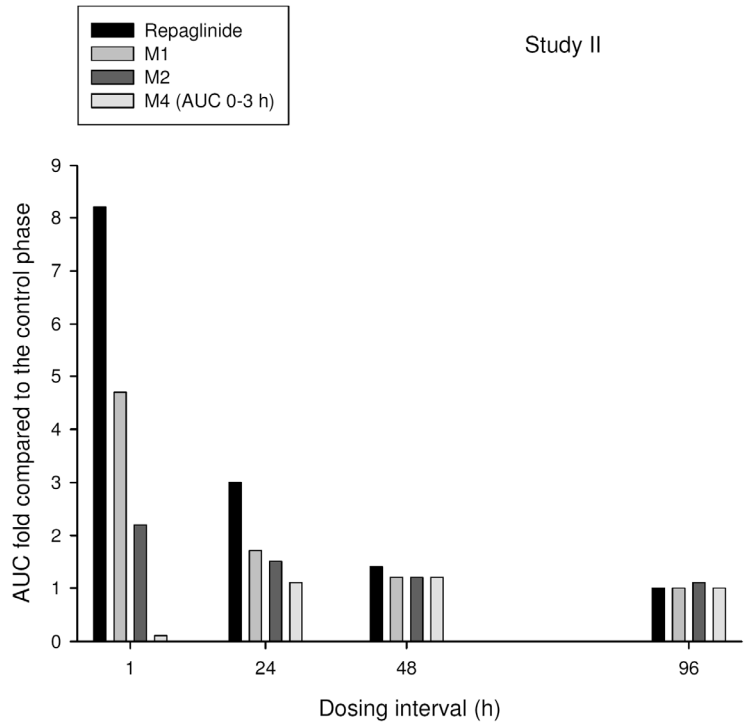

Figure 10. Fold changes in the AUC of repaglinide and its metabolites compared to the control phase, when the last dose of gemfibrozil was taken 1, 24, 48 or 96 h before repaglinide intake. For repaglinide M4, AUC_{0-3h} data is presented. For all other compounds, $AUC_{0-\infty}$ data is presented.

The $AUC_{0-\infty}$ of CYP3A4-dependent repaglinide metabolite M1 was 4.7-, 1.7-, 1.2- and 1.0-fold compared to the control phase, when the last dose of a 3-day gemfibrozil pretreatment with 600 mg twice daily was taken 1, 24, 48 and 96 h before repaglinide, respectively (Figure 10; $P < 0.05$ in the 48 h phase, $P < 0.001$ in the 1 and 24 h phases). The $AUC_{0-\infty}$ of repaglinide M2 was 2.2-, 1.5-, 1.2- and 1.1-fold compared to the control phase, respectively (Figure 10; $P < 0.05$ in the 1 and 24 h phases). The formation of the CYP2C8-dependent repaglinide metabolite M4 was significantly impaired only in the shortest 1 h dosing interval. The AUC_{0-3h} of M4 was 11% and the C_{max} 8% of the control value, when the last dose of gemfibrozil was taken 1 h before repaglinide, respectively (Figure 10; $P < 0.001$). The M4/repaglinide AUC_{0-3h} ratio was 2%, 40%, 90% and 100% of control in the 1, 24, 48 and 96 h interval phases, respectively ($P < 0.001$ in the 1 and 24 h dosing intervals).

The gemfibrozil and gemfibrozil 1-O-β-glucuronide concentrations at the time of repaglinide intake were approximately 1%, 0.1% and 0.02% of the C_{max} values estimated in the 1 h dosing interval phase, when repaglinide was taken 24, 48 and 96 h after the last gemfibrozil dose.

With a mechanism-based inhibition model assuming termination of the CYP2C8 inactivating process within 24 h after the last gemfibrozil dose, a constant rate of CYP2C8 enzyme production and a first-order process of enzyme degradation, the mean first-order degradation rate of CYP2C8 was estimated at 0.00056 min^{-1} and the corresponding $t_{½}$ at 22 h, when each subject was analysed separately.

3. Dose-dependency of CYP2C8 inhibition with gemfibrozil (III and IV)

Study III. The interaction between gemfibrozil and repaglinide was shown to be dose-dependent. The results were consistent with approximately 50% inhibition of CYP2C8 already with a single 30 mg dose of gemfibrozil, and > 90% CYP2C8 inhibition with a 900 mg dose.

The geometric mean $AUC_{0-\infty}$ of repaglinide was 1.7-, 4.3-, 6.5- and 8.1-fold compared to control (i.e., with placebo) when repaglinide was taken 1 h after a single oral dose of 30, 100, 300 and 900 mg gemfibrozil, respectively (Figure 11, Table 16; $P < 0.001$). The geometric mean C_{max} of repaglinide increased by 1.3-, 1.6-, 2.0- and 2.3-fold, respectively ($P < 0.05$).

The concentrations of M1 increased with increasing gemfibrozil doses. The $AUC_{0-\infty}$ of repaglinide M1 was 1.1-, 1.9-, 2.9- and 4.3-fold in the 30, 100, 300 and 900 mg phases, compared to the control phase, respectively (Figure 11; $P < 0.001$ in the 100, 300 and 900 mg phases). The $AUC_{0-\infty}$ of repaglinide M2 was 1.0-, 1.1-, 1.2- and 1.5-fold compared to control, respectively ($P < 0.05$ with 300 and 900 mg doses). The formation of the CYP2C8-dependent repaglinide metabolite M4 was significantly impaired by gemfibrozil pretreatment. In the 900 mg phase, M4 could not be detected in the samples of most subjects, and pharmacokinetic variables could not be calculated. The geometric mean AUC_{0-3h} of repaglinide M4 was 102%, 50% and 30% of control when taken 1 h after a single oral dose of 30, 100 and 300 mg gemfibrozil, respectively ($P < 0.05$ in the 100 mg phase, $P < 0.001$ in the 300 mg phase). The M4/repaglinide AUC_{0-3h} ratio was 66%, 22%, and 11% of the control in the 30, 100 and 300

mg phases, respectively ($P < 0.005$ in the 30 mg phase, $P < 0.001$ in the 100 and 300 mg phases).

The AUC values of gemfibrozil and its glucuronide increased slightly more than dose-proportionally. The nonlinearity was slightly greater for the glucuronide than for the parent gemfibrozil, resulting in a dose-dependent increase in the glucuronide/gemfibrozil AUC-ratio. With the power-model approach using the equation $AUC_{0-\infty} = e^{\alpha} \cdot dose^{\beta}$, β was estimated at 1.21 (95% confidence interval (CI) 1.15, 1.26) for gemfibrozil and at 1.33 (95% CI 1.28, 1.39) for gemfibrozil 1-O-β-glucuronide.

A mechanism-based inhibition model of CYP2C8 with gemfibrozil 1-O-β-glucuronide $C_{avg,10h}$ could well explain the observed extent of the interaction (repaglinide $AUC_{inhibited}/AUC_{control}$) in pooled data (Table 17; $r^2 = 0.79$). Using this model, the fraction of the repaglinide dose metabolised by CYP2C8 ($f_{m,CYP2C8}$) and the ratio of unbound hepatocyte concentration to total plasma concentration ($C_{h,u}/C_{p,tot}$) of gemfibrozil 1-O-β-glucuronide were estimated at 89% and 0.24, respectively. When the same model was applied to each subject separately, the estimates for the $f_{m,CYP2C8}$ of repaglinide and the $C_{h,u}/C_{p,tot}$ of gemfibrozil 1-O-β-glucuronide averaged 89% and 0.28, respectively.

With a model combining mechanism-based CYP2C8 inhibition and reversible OATP1B1 inhibition model, a slightly better fit to the observed *in vivo* interaction data could be achieved (Table 17; $r^2 = 0.81$). With this model, the estimated $f_{m,CYP2C8}$, $C_{h,u}/C_{p,tot}$ -ratio and $f_{t,OATP1B1}$ were 84%, 0.37 and 94%, respectively. It was estimated that ~50% inhibition of CYP2C8 can be reached already with a single dose of 30 mg gemfibrozil, and > 90% inhibition by administering a single 900 mg dose of gemfibrozil.

Table 16. Repaglinide geometric mean $AUC_{0-\infty}$ fold by gemfibrozil dosing scheme, when the only/last gemfibrozil dose was given 1 h before repaglinide.

Gemfibrozil dosing scheme (study)	*Repaglinide geometric mean $AUC_{0-\infty}$ fold increases with different gemfibrozil doses (and estimates of the level of CYP2C8 inhibition based on M4/repaglinide AUC ratios)*				
	30 mg	*100 mg*	*300 mg*	*600 mg*	*900 mg*
Single dose (III)	1.7 (34%)	4.3 (78%)	6.5 (89%)	-	8.1 (> 95%)
Twice daily for 5 days (IV)	3.4 (72%)	5.5 (91%)	-	7.0 (> 95%)	-

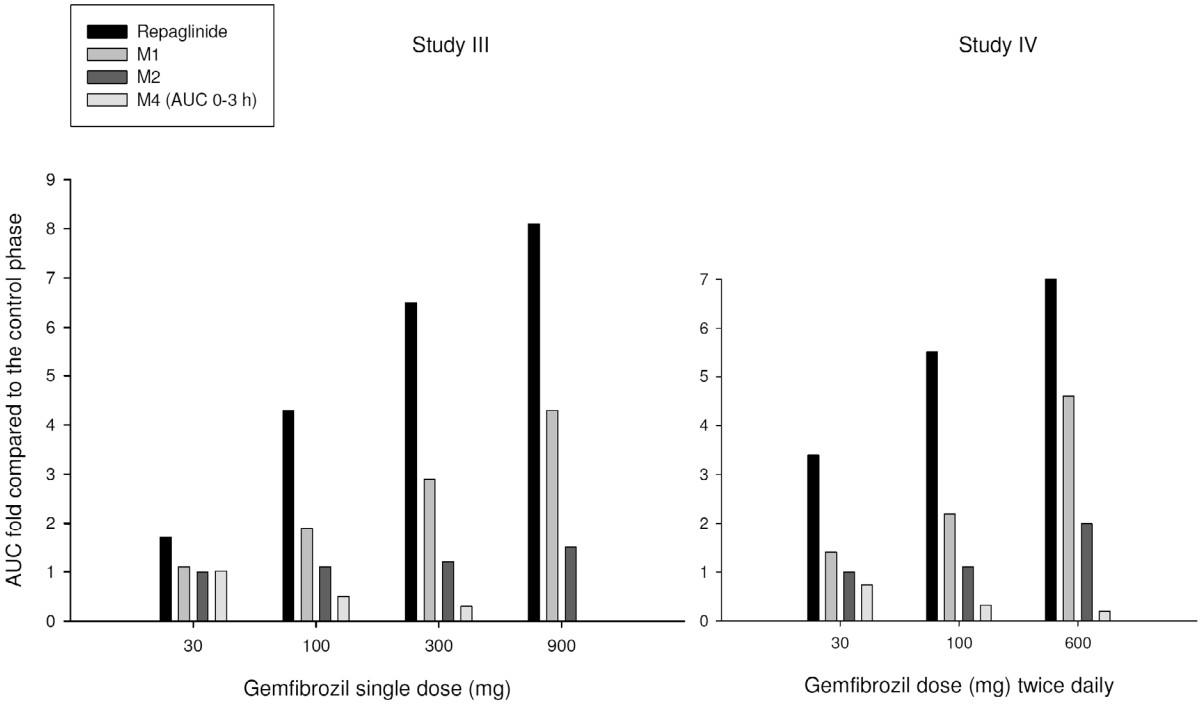

Figure 11. Fold changes in the AUC of repaglinide and its metabolites compared to the control phase, when repaglinide was taken 1 h after a single 30, 100, 300 or 900 mg dose of gemfibrozil (Study III, left panel) or 1 h after the last dose of 30, 100 or 600 mg gemfibrozil twice daily (Study IV, right panel). For repaglinide M4, $AUC_{0\text{-}3h}$ data is presented. For all other compounds, $AUC_{0\text{-}\infty}$ data is presented.

Study IV. When gemfibrozil was administered twice daily for 5 days, large increases in the AUC of repaglinide were observed already with small gemfibrozil doses. Repaglinide geometric mean $AUC_{0\text{-}\infty}$ was increased by 3.4-, 5.5- and 7.0-fold of control after a 5-day pretreatment with gemfibrozil 30, 100 or 600 mg twice daily (Figure 11, Table 16; $P < 0.001$). The geometric mean C_{max} of repaglinide increased by 1.5-, 1.9- and 2.0-fold, respectively ($P < 0.005$).

The $AUC_{0\text{-}\infty}$ of M1 was increased by 1.4-, 2.2- and 4.6-fold in the 30, 100 and 600 mg gemfibrozil phases, respectively (Figure 11; $P < 0.05$). The $AUC_{0\text{-}\infty}$ of repaglinide M2 was 1.0-, 1.1- and 2.0-fold compared to the control phase, respectively ($P < 0.001$ in the 600 mg phase). The $AUC_{0\text{-}3h}$ of M4 was decreased to 74% (non-significant), 32% and 20% of control ($P < 0.001$), respectively. The M4/repaglinide $AUC_{0\text{-}3h}$ ratio was 28%, 9%, and 5% of control in the 30, 100 and 600 mg phases, respectively ($P < 0.001$ in all phases).

With the 100 mg and 600 mg gemfibrozil doses (which are 3.3- and 20-fold compared to the 30 mg dose, respectively) the $AUC_{0\text{-}10h}$ of gemfibrozil was 3.9- and 31.9-times higher than with the smallest 30 mg gemfibrozil dose, i.e., the AUC increased more than dose-proportionally. The $AUC_{0\text{-}10h}$ of gemfibrozil 1-O-β-glucuronide increased even more, to 4.7- and 52.8-fold, respectively. With the power-model approach using the equation $AUC_{0\text{-}12h} = e^{\alpha} \cdot dose^{\beta}$, β was estimated at 1.16 (95% CI 1.07, 1.25) for gemfibrozil and at 1.33 (95% CI 1.24, 1.42) for gemfibrozil 1-O-β-glucuronide.

A static mechanism-based inhibition model using $C_{avg,12h}$ of gemfibrozil 1-O-β-glucuronide as the inhibitor concentration was fitted to the observed repaglinide AUC ratio data. With non-linear regression analysis $f_{m,CYP2C8}$ was predicted at 86% and $C_{h,u}/C_{p,tot}$ of gemfibrozil 1-O-β-glucuronide at 0.77 (Table 17; r^2 = 0.634). The CYP2C8 mechanism-based inhibition model was also applied to individual data, which yielded a mean $f_{m,CYP2C8}$ of repaglinide of 86% and $C_{h,u}/C_{p,tot}$ of gemfibrozil 1-O-β-glucuronide of 0.86. Based on this analysis > 50% inhibition of CYP2C8 was obtained with the 30 mg gemfibrozil dose in all the subjects and at least 90% inhibition was reached with the 100 mg dose in 8 of the 10 subjects.

Table 17. Predicted values of unknown parameters by the best fitting models in studies III and IV.

Study	*Model*	*Repaglinide* $f_{m,CYP2C8}$	*Repaglinide* $f_{t,OATP1B1}$	*Gemfibrozil 1-O-β-glucuronide* $C_{h,u}/C_{p,tot}$	r^2
Single dose	CYP2C8 MBI	89%	-	0.24	0.792
Single dose	CYP2C8 MBI + competitive OATP1B1	84%	94%	0.37	0.805
Multiple dose	CYP2C8 MBI	86%	-	0.77	0.634
Multiple dose	CYP2C8 MBI + competitive OATP1B1	85%	15%	0.86	0.635

$f_{m,CYP2C8}$, fraction metabolised by CYP2C8; $f_{t,OATP1B1}$, fraction transported by OATP1B1; $C_{h,u}/C_{p,tot}$, ratio of unbound hepatocyte concentration to total plasma concentration; r^2, coefficient of determination

As in study III, a combined CYP2C8 mechanism-based inhibition - competitive OATP1B1 inhibition model using gemfibrozil 1-O-β-glucuronide $C_{avg,12h}$ and C_{max}, respectively, as inhibitor concentration yielded a slightly better fit (r^2= 0.635) than the model assuming mechanism-based inhibition alone (Table 17). With this combination model, the $C_{h,u}/C_{p,tot}$, $f_{m,CYP2C8}$ and $f_{t,OATP1B1}$ were predicted at 0.86, 85% and 15%, respectively.

With a mechanism-based inhibition model based on the mean $C_{h,u}/C_{p,tot}$ obtained in study IV, fold increases in the AUC of drugs with different $f_{m,CYP2C8}$ and the remaining CYP2C8 activity were estimated for different gemfibrozil doses taking into account the dose-proportionality of the AUC of gemfibrozil 1-O-β-glucuronide as described by the power model used. It was estimated that a 50% inhibition of CYP2C8 can be reached already by twice-daily administration of << 30 mg gemfibrozil, and a > 90% inhibition by administering 100 mg gemfibrozil twice daily.

DISCUSSION

1. Methodological considerations

1.1. Study design

The studies were carried out as *in vivo* clinical drug-drug interaction studies with a randomised, balanced, cross-over design. All subjects in the studies acted as their own controls. This was particularly important for getting reliable data on the extent of the interaction, as the pharmacokinetics of repaglinide, the probe drug used in these studies, has been shown to be highly variable between individuals (Niemi *et al*., 2003c).

The studies examining the effect of the gemfibrozil dose (III and IV) were of placebo-controlled design. Both the subjects and the investigators remained blinded during the clinical phase of the studies. Studies I and II assessing the effect of timing of gemfibrozil administration included also a control phase (i.e., no gemfibrozil was administered), but placebos were not used because the blinding would have required placebo administration at multiple time points, including night time administration in study I, in the gemfibrozil phases as well as in the control phase. In studies of 5 different schemes (intervals) of study drug administration, the use of placebos, and the burden of study related activities to the subjects, would have been too extensive. As the main results of these studies are based on pharmacokinetics, and only to a minor extent on pharmacodynamic variables, they can be considered reliable also without the use of placebo. Subjective assessments, e.g., symptoms of hypoglycaemia, which could be affected by the information of the actual study treatment, were used mainly for safety measures, not as the primary results of the studies.

Both the slow recovery of enzyme activity due to mechanism-based inhibition and the half-lives of the study drugs were considered when the wash-out periods in the studies were determined. To prevent carry-over effect, long (2-3 weeks in minimum) wash-out periods were held in all studies.

Since the CYP2C8 and OATP1B1 inhibitory effects of gemfibrozil and gemfibrozil 1-O-β-glucuronide have already been extensively studied *in vitro*, no *in vitro* studies were included in this work. Important inhibition constants such as k_{inact}, K_I, IC_{50} have been previously reported by other groups (Shitara *et al*., 2004; Ogilvie *et al*., 2006). However, substantial amount of information needed for the *in vitro* - *in vivo* extrapolation of the mechanism-based inhibition of CYP2C8 by gemfibrozil were missing. In addition, other information such as data of appropriate design of clinical drug-drug interaction studies and safer therapeutic use of drugs could be obtained with these *in vivo* studies.

1.2. Ethical and safety considerations

In the studies, gemfibrozil and repaglinide, drugs with potential pharmacological effects were administered to healthy volunteers. This can be justified by many arguments. Apart from the

potential for drug interactions, treatment with the lipid-lowering agent gemfibrozil has been well-tolerated and safe in clinical use. In our studies, single doses or only short, up to 5-day courses of gemfibrozil were used. The maximal doses used in the studies were not higher than the approved therapeutic doses of gemfibrozil, 900 mg once or 600 mg twice daily. Although concomitant use of gemfibrozil and the blood glucose lowering oral antidiabetic agent repaglinide is contraindicated in many countries, repaglinide was used as an *in vivo* probe for measuring CYP2C8 activity in the subjects. For safety reasons, the repaglinide dose was small, 0.25 mg, in all studies. It has been argued that the metabolism of repaglinide could differ at different doses, but this is not considered a concern in this work as the same repaglinide dose was used in all study phases. In addition, at least in one study, the metabolism of repaglinide has been shown to be unaltered over a wide dose range (Kalliokoski *et al.*, 2008c). Further, in some cases, based on safety grounds, the use of smaller than clinical doses of the drugs are considered adequate for drug-drug interaction studies (Huang *et al*., 2007).

The safety of the subjects was carefully followed in all studies. Volunteers with any signs of hepatic or kidney function abnormalities or other medically significant abnormalities were excluded from the studies. The subjects entering the studies were given proper guidance concerning, e.g., avoiding physical exercise before and in the evenings of the study days. Female subjects were routinely ascertained not to be pregnant before and during the studies, and also in case of a suspected possibility of pregnancy. During the study days the subjects were under direct medical supervision. Rescue medication and equipment were available for medical emergency situations. Blood glucose levels were measured frequently after repaglinide intake with predefined rules of actions in case of hypoglycaemia. The subjects were allowed to leave the study premises in the evening of the study days only after blood glucose had reached the predefined safety level. In case of adverse reactions, the subjects were rigorously followed up by the investigators.

1.3. Selection of *in vivo* probe drug for CYP2C8 activity

Repaglinide was chosen as the probe drug for measuring *in vivo* CYP2C8 activity. Repaglinide was considered as a proper *in vivo* probe for CYP2C8 activity because it is metabolised mainly by CYP2C8. CYP3A4 has also been shown to catalyse repaglinide metabolism, but it is not inhibited by gemfibrozil or its glucuronide (Backman *et al*., 2000; Shitara *et al*., 2004; Kajosaari *et al*., 2005a). In addition, the $t_{1/2}$ of repaglinide is short, which makes it superior compared to other potential CYP2C8 probe drugs for studying time-related changes in enzyme activity. Repaglinide is also recommended to be used as a probe drug for CYP2C8 activity for *in vivo* studies by the regulatory authorities (FDA, 2006; EMA, 2010), although the blood glucose lowering effect can expose the subjects to hypoglycaemia. Compared to other *in vivo* probes for CYP2C8 activity, repaglinide has shown the best sensitivity for CYP2C8 activity. Pioglitazone and rosiglitazone have been less sensitive CYP2C8 probes than repaglinide (Lai *et al*., 2009). The use of other CYP2C8 probes was not considered reasonable either. Cerivastatin has been withdrawn from the market, which impairs its accessibility. Paclitaxel, an antineoplastic agent, cannot be used in healthy subjects due to safety reasons. Therefore, in spite of its hypoglycaemic effect, repaglinide was chosen as the probe drug for this work. The prevention, detection and management of the

hypoglycaemic effect of repaglinide were taken carefully into account in the studies, as discussed earlier. Recently, montelukast was shown to be metabolised mainly through CYP2C8 *in vivo*. Due to its better safety profile, similar sensitivity and maybe less complex interaction potential, montelukast may replace repaglinide as a probe drug for CYP2C8 activity in the future (Karonen *et al*., 2011; VandenBrink *et al*., 2011).

1.4. Pharmacokinetic modelling

The gemfibrozil dose-proportionality was assessed using the power-model approach, which is currently considered as the method of choice (Sheng *et al*., 2010). In the single-dose study (III), the gemfibrozil and gemfibrozil 1-O-β-glucuronide $AUC_{0-\infty}$ data were used in the modelling, whilst for the data from the multiple dose study (IV) extrapolations of the AUC of the dosing interval (AUC_{0-12h}) calculated using the last measured concentration C_{10h} and k_e were utilised.

The static inhibitory models used for the data obtained in studies III and IV have been developed for predicting interactions in steady state conditions. Based on the half-life of gemfibrozil and its glucuronide, a steady state was achieved in study IV with a 5-day course of gemfibrozil. On the contrary, in study III using a single dose of inhibitor, a steady state was not achieved. Therefore, the application of the steady state models to this data could be argued. However, when comparing the findings of studies III and IV, the only difference was that the $C_{h,u}/C_{p,tot}$ ratio was 3.2 times bigger in study IV than in study III indicating that the strong inhibition of CYP2C8 was obtained with lower gemfibrozil 1-O-β-glucuronide concentrations and lower gemfibrozil doses in study IV than in study III. Thus, the main result, the estimate of CYP2C8 inhibition with different gemfibrozil doses, was in line with the expectations that a steady state of mechanism-based inhibition is reached slowly with low inhibitor doses. Although physiologically based pharmacokinetic (PBPK) modelling of drug interactions in general provides advantages compared to static models by taking the changes in inhibitor, substrate and enzyme concentration over time into account, for mechanism-based inhibition, static models can be even more predictive than dynamic models (Einolf, 2007). However, as the more sophisticated dynamic, PBPK models are currently rapidly evolving, this may change in the future.

In the models, previously published CYP2C8 and OATP1B1 inhibition constants for gemfibrozil and gemfibrozil 1-O-β-glucuronide were used. Corrections for microsomal binding were used, when appropriate. For OATP1B1 inhibition, a published IC_{50} for cerivastatin uptake was used, as inhibition constants for repaglinide uptake were not available. As OATP1B1 inhibition by gemfibrozil has been shown to be substrate-dependent (Noé *et al*., 2007), better estimates in the analyses could have been achieved by the use of repaglinide specific inhibition constants. The OATP1B1 inhibition constant (for cerivastatin) used in the modelling was obtained by halving the IC_{50} value, in order to avoid underestimation of the contribution of OATP1B1 inhibition, if any, from the *in vivo* AUC ratio data (Hinton *et al*., 2008). Despite these facilitative approaches, the OATP1B1 inhibition was estimated to be a minor contributor in the observed repaglinide-gemfibrozil interaction. This provides further evidence that this interaction is mainly due to inhibition of CYP2C8.

1.5. Generalisation of the data obtained

Further, it can be argued whether the data obtained from these drug-drug interaction studies conducted in healthy volunteers can be generalised to patients. Drug metabolism and pharmacokinetics may indeed be altered by at least certain pathologic conditions, e.g., hepatic diseases or diabetes (Palatini *et al*., 2010; De Moraes *et al*., 2011). According to the current knowledge, the effects of reversible inhibition may be diminished, but the effects of mechanism-based inhibition are mostly retained in the patients with hepatic diseases (Palatini *et al*., 2010). In addition, the generalisation of the data to other CYP2C8 substrates could be questioned. The CYP2C8 inhibitory potential was suggested to be the most important factor in the repaglinide-gemfibrozil interaction with only a small contribution of OATP1B1 inhibition. Therefore, besides for IVIVE and the design of clinical CYP2C8-mediated drug-drug interaction studies, the data from these studies are considered adequate for guiding the management of drug-drug interaction potential of other CYP2C8 substrates in clinical use.

2. Effect of time on mechanism-based inhibition of CYP2C8 by gemfibrozil

In most previous clinical drug-drug interaction studies with gemfibrozil and repaglinide, the last dose of gemfibrozil pretreatment had been given 1 h before repaglinide. Simultaneous or very close administration of the inhibitor and substrate drug is adequate for achieving and maintaining the inhibitor concentrations during the exposure to the substrate drug. However, mechanism-based inhibition requiring a catalytic step before inactivation has been believed to occur more slowly than reversible inhibition. For studying the onset of mechanism-based CYP2C8 inhibition single doses of gemfibrozil 600 mg were given to healthy volunteers simultaneously with, and 1, 3 and 6 h before repaglinide. The pharmacokinetic variables of repaglinide and its metabolites (especially the M4/repaglinide $AUC_{0\text{-}3h}$ ratio) showed a rapid onset of CYP2C8 inhibition. Repaglinide geometric mean $AUC_{0\text{-}\infty}$ increased by 5.0-, 6.3-, 6.6- and 5.4-fold and C_{max} by 1.4-, 2.1-, 2.1- and 2.0-fold compared to control when gemfibrozil was given simultaneously with, or 1, 3 or 6 h before repaglinide. These and the pharmacokinetic variables of repaglinide metabolites suggested that the interaction between gemfibrozil and repaglinide develops in 1 h after a single dose of gemfibrozil reaching its maximum with a time interval of 1-3 h. This rapid onset was surprising, as several steps, e.g., absorption, transport and metabolism of gemfibrozil and a catalytic cycle of gemfibrozil 1-O-β-glucuronide by CYP2C8 are required before the inhibition of the enzyme can occur. In fact the data showed already some recovery of the enzyme activity when the dosing interval was 6 h. The information concerning the development of mechanism-based inhibition in humans can be used in the design of future clinical drug-drug interaction studies. Also, this rapid onset of a potentially hazardous interaction needs to be recognised for safer use of drugs in patients.

Before this study, the inhibitory effect of gemfibrozil on repaglinide metabolism had shown to persist at least 12 h after the last gemfibrozil dose (Tornio *et al*., 2008a). This finding provoked the idea of a study design of even longer dosing intervals. In study II, dosing intervals of 1, 24, 48 and 96 h were used. The geometric mean AUC of repaglinide was 8.2-, 3.0-, 1.4- and 1.0-fold compared to control, respectively; i.e., repaglinide metabolism was shown to be recovered back to the control level in the longest 96 h dosing interval. This was

supported also by the M4/repaglinide AUC data, which reflects the remaining CYP2C8 activity. From the data from study II, the first-order degradation rate constant k_{deg} and $t_{1/2}$ of CYP2C8 could be calculated from human *in vivo* data for the first time. Previously, only *in vitro* estimations of these variables had been published. The $t_{½}$ calculated from study II (22 h) was in good agreement with the corresponding *in vitro* estimations (8-41 h (Renwick *et al*., 2000)). In addition, the k_{inact}/k_{deg} ratio of mechanism-based CYP2C8 inhibition by gemfibrozil 1-O-β-glucuronide can be estimated at 380 based on the k_{deg} obtained. The *in vivo* k_{deg} for CYP2C8 can be used in future *in vitro - in vivo* extrapolations of interactions caused by the mechanism-based inhibition of CYP2C8.

Based on these studies assessing the effects of dosing time on CYP2C8 inhibition caused by gemfibrozil, suggestions for better tolerated concomitant clinical use of gemfibrozil and CYP2C8 substrate drugs in patients could be given. If CYP2C8-dependent substrate drugs, particularly those with a narrow therapeutic index (if the use with gemfibrozil is not contraindicated), are started during the first 1-3 days after stopping gemfibrozil treatment, the reduction of their doses should be considered. However, it should be kept in mind that, e.g., the concomitant use of gemfibrozil and repaglinide is contraindicated in patients in many countries.

3. Dose-dependency of mechanism-based inhibition of CYP2C8 by gemfibrozil

With increasing oral gemfibrozil doses the AUC of gemfibrozil and gemfibrozil 1-O-β-glucuronide increased more than dose-proportionally. The β value in the power model approach equation $AUC = e^{\alpha} \cdot dose^{\beta}$ was estimated at 1.21 and 1.16 for gemfibrozil in the single (III) and multiple (IV) dose studies, respectively. The corresponding value for gemfibrozil 1-O-β-glucuronide was estimated at 1.33 in both studies. This finding of more than dose-proportional pharmacokinetics may not have any clinical implications, but can be used in the estimations of the inhibitory potential of different gemfibrozil doses and in the design of clinical drug-drug interaction studies.

The repaglinide AUC increased dose-dependently by increasing doses of gemfibrozil both in the single (III) and multiple (IV) dose study. The repaglinide geometric mean $AUC_{0-\infty}$ was increased by 1.7-, 4.3-, 6.5- and 8.1-fold compared to control when given 1 h after a single dose of 30, 100, 300 or 900 mg of gemfibrozil. The fold change observed in the highest 900 mg dose phase was of the same extent as seen in previous studies with 600 mg twice daily for 3 days, i.e., almost a maximal interaction level could be obtained. In the multiple dose study, repaglinide geometric mean $AUC_{0-\infty}$ increased by 3.4-, 5.5- and 7.0-fold of control after a 5-day pretreatment with gemfibrozil 30, 100 or 600 mg twice daily.

The repaglinide-gemfibrozil interaction, as described by the increase in repaglinide AUC and the decrease in the M4/repaglinide AUC ratio, is more potent during multiple dosing of gemfibrozil. This can be seen particularly in the smallest dose range studied (30-100 mg). This reflects the gradual increase in the loss of enzyme activity, which is typical for mechanism-based inhibition. At the largest gemfibrozil dose range such a great increase in the

extent of the interaction cannot be seen, as the large gemfibrozil doses are able to cause almost a maximal interaction already at single doses, and therefore, more potent inhibition during multiple dosing is not possible.

When different individual inhibition models were fitted to the observed interaction data from both studies, the best fit was obtained by the mechanism-based inhibition model. A slightly better fit was obtained for the data in the single dose study than in the multiple dose study (r^2=0.79 vs. 0.63), although the models have been developed to be used for steady state situations. For the data from both studies, a slightly better fit to the models could be obtained by adding a competitive OATP1B1 inhibition component to the CYP2C8 mechanism-based inhibition model. In both models concentration of gemfibrozil 1-O-β-glucuronide was used as the inhibitor concentration.

Several predictions were made using different inhibition mechanism models both in separate and combined manner by using different inhibitor concentrations in the equations. Models using gemfibrozil 1-O-β-glucuronide as the inhibitor yielded better fits than those using gemfibrozil as the inhibitor concentration. This could be expected, because the inhibitory effect of gemfibrozil on CYP2C8 has been shown to be much weaker than that of gemfibrozil 1-O-β-glucuronide (Shitara *et al*., 2004). Interestingly, for CYP2C8 inhibition mechanisms, better fits to the data were obtained by using C_{avg} than C_{max} in the equations. This was also the case in the models assuming mechanism-based inhibition, although in literature, for mechanism-based inhibition, C_{max} has been reported to be more predictive than C_{avg} (Obach *et al*., 2007; Obach, 2009).

Based on these models, the fraction of the repaglinide dose metabolised by CYP2C8 was 84-89% in the studies. This is somewhat more than estimated in previous studies (0.49-0.61) (Hinton *et al*., 2008; Baer *et al*., 2009), but in good agreement with the profound *in vivo* increases in repaglinide AUC after treatment with a strong CYP2C8 inhibitor gemfibrozil. The repaglinide fraction transported by OATP1B1 was estimated at 94% and 15% based on the data of the single and multiple dose studies. With single dosing, the OATP1B1 inhibition contributes to some extent to the observed interaction. However, when multiple dosing is used the mechanism-based inhibition of CYP2C8 is so strong that the contribution of OATP1B1 inhibition is smaller. The differences in the estimates of $f_{t,OATP1B1}$ can also be explained by the low specificity of the model for OATP1B1 inhibition. The IC_{50} value of cerivastatin uptake instead of that of repaglinide uptake was used and the reported IC_{50} value of cerivastatin was halved in order to avoid underestimation. A repaglinide specific IC_{50} value, if available, would therefore make the predictions more precise. In addition, when analysing more than one inhibition method concomitantly, a dynamic model might also improve the predictions.

The gemfibrozil 1-O-β-glucuronide $C_{h,u}/C_{p,tot}$ was predicted at 0.24-0.86 with the best fitting models in the studies, i.e., the total plasma concentrations of gemfibrozil 1-O-β-glucuronide would be 1-4 -fold compared to its hepatic unbound concentrations. As gemfibrozil 1-O-β-glucuronide is known to concentrate in the liver in rats (Sallustio *et al*., 1996), and considering the plasma fraction unbound (f_u; 0.115) of gemfibrozil 1-O-β-glucuronide, this level of accumulation can be regarded as possible. When other than CYP2C8 mechanism-based inhibition models were used, the predicted $C_{h,u}/C_{p,tot}$ values were much higher. In that

case higher intrahepatic gemfibrozil 1-O-β-glucuronide concentrations are needed for potent inhibition. The estimate of $C_{h,u}/C_{p,tot}$ based on the mechanism-based inhibition model was 3.2 times higher in the multiple dose study (IV) than in the single dose study (III). The estimate based on study IV is likely to be more reliable, because the design in study III violated the assumption of steady-state conditions, while the inhibitor concentrations in study IV were very close to steady-state. If the $C_{h,u}/C_{p,tot}$ estimated in study IV was true, the average unbound gemfibrozil 1-O-β-glucuronide concentrations in the hepatocyte would be approximately 14 µM with twice daily dosing of 600 mg gemfibrozil. After administration of a single 600 mg gemfibrozil dose, the unbound gemfibrozil 1-O-β-glucuronide concentrations in the hepatocyte are likely to be almost as high as after multiple dosing. According to *in vitro* data (Niemi *et al*., 2003b; Ogilvie *et al*., 2006), a 10 µM gemfibrozil 1-O-β-glucuronide concentration in the hepatocytes could lead to ~90% inactivation of CYP2C8 in 40-120 minutes, which is sufficiently rapid to explain the rapid inactivation observed after a single 600 mg gemfibrozil dose in Study I. Therefore, the results from these studies assessing both time and dose relationships of mechanism-based inhibition of CYP2C8 by gemfibrozil are in good agreement with each other.

The mechanism-based inhibition of CYP2C8 by gemfibrozil was shown to be clearly dose-dependent. With increasing gemfibrozil doses, both in the single and multiple dosing schemes, the extent of the inhibition increased. A level of ~50% inhibition of the CYP2C8 activity could already be obtained by a single 30 mg dose of gemfibrozil or by twice daily administration of << 30 mg of gemfibrozil. > 90% of the CYP2C8 activity could be inhibited by a single dose of 900 mg or twice daily dosing of 100 mg gemfibrozil.

4. General discussion and future prospects

Drug-drug interactions may cause severe consequences on drug efficacy and patient safety. Therefore, the detection, evaluation, understanding and management of the drug-drug interaction potential of therapeutic agents are essential. Drug-drug interaction potential can currently be studied by *in vitro*, *in vivo* and *in silico* methods. Of several possible mechanisms leading to drug-drug interactions, the interactions based on metabolic enzyme inhibition are often regarded as of great clinical importance.

The clinical importance of metabolic enzyme inhibition is based on, e.g., the fraction of the victim drug metabolised with the enzyme concerned (f_m) and the therapeutic index of the victim drug. Drugs which are mainly metabolised with the inhibited enzyme and which have a narrow therapeutic index may cause severe adverse reactions in clinical use, when administered concomitantly with an enzyme inhibitor. The knowledge of metabolic pathways of the drugs and their relative fractions are therefore important in drug development. In *in vitro - in vivo* extrapolations, the ratio of clinical drug concentrations and the concentration supporting half of the maximal inhibition is essential.

In the case of mechanism-based inhibition, the extrapolation of *in vivo* consequences is even more complex. The fraction metabolised with the enzyme concerned, therapeutic index and the *in vivo* drug concentrations of the substrate drug, as well as the inhibitor potency are

important also in this interaction mechanism. In addition, the ratio of the rate of enzyme inactivation (k_{inact}) and the first order degradation rate of the inhibited enzyme (k_{deg}) impact the clinically observed interaction. A high k_{inact}/k_{deg} ratio can make the mechanism-based inhibition clinically significant, although the $[I]/K_I$ ratio would be fairly small.

Before this thesis work, gemfibrozil was recognised as an important CYP2C8 inhibitor; it was recommended as a model inhibitor of CYP2C8 for drug development (FDA, 2006). Also, the importance of CYP2C8-mediated interactions in clinical drug therapy had recently been acknowledged, as the list of CYP2C8 substrate drugs has continuously increased. The mechanism-based nature of CYP2C8 inhibition by gemfibrozil 1-O-β-glucuronide and the extent of repaglinide-gemfibrozil interaction were already known (Niemi *et al*., 2003b; Ogilvie *et al*., 2006). The *in vivo* time and dose relationships of this interaction, as those of many interactions caused by mechanism-based inhibition, were fairly unexplored.

This work provides new data concerning the turnover half-life of CYP2C8 and the onset, persistence and dose-dependency of CYP2C8 inhibition by gemfibrozil, which will help the *in vitro –in vivo* extrapolation of drug interactions caused by the mechanism-based inhibition of CYP2C8 and guide the design of clinical drug-drug interactions, at least with regard to the dosing and timing of gemfibrozil administration.

Based on the pharmacokinetic modelling made in this work mechanism-based CYP2C8 inhibition was suggested to be the main component behind repaglinide-gemfibrozil interaction. The data of two dose-related studies were modelled separately, and mechanism-based inhibition was proposed to be the main component by the results of both of them. However, the results of these studies propose small additional effects of other mechanisms as causal components in the observed clinical interaction. With a static model using only data from one study separately the different interaction mechanism could not be quantified very well. One reason for this is probably the fact that the inhibitory potencies of the mechanisms are different, i.e., the contribution of the less-potent mechanisms could not be separated and quantified behind the mechanism-based inhibition of CYP2C8. The modelling of the interaction mechanism would be more powerful and provide more precise data, if data from different repaglinide-gemfibrozil studies with different dosing and timing schemes would be pooled, and analysed together using dynamic, physiologically-based pharmacokinetic models.

The repaglinide-gemfibrozil interaction was proposed to be mainly due to the effects of gemfibrozil 1-O-β-glucuronide, the metabolite of gemfibrozil. In addition to the suggestion of mechanism-based inhibition being the main component of this interaction, this work provides confirmation of the importance of safety evaluation of metabolites, an issue which has gained attention in drug development (Baillie *et al*., 2002; Atrakchi, 2009; EMA, 2010; Parkinson *et al*., 2010; VandenBrink and Isoherranen, 2010).

This work has also clinical implications. To enable safe use of drugs, information concerning the onset and persistence of drug-drug interactions is crucial. Although the clinical use of gemfibrozil has decreased, there are still patients, who may benefit from the lipid-lowering effects of gemfibrozil. Safety margins for starting and stopping concomitant administration of gemfibrozil and CYP2C8 substrates were obtained by this work, which further supports

earlier findings that caution should be exercised when gemfibrozil is used concomitantly with substrates of CYP2C8. It should be noted, that concomitant administration of gemfibrozil and some CYP2C8 substrates such as repaglinide is contraindicated in some countries.

One additional future prospect of this work is related to the intentional modification of pharmacokinetics of drugs. The bioavailability of drugs with substantial CYP2C8-mediated first-pass metabolism could be increased by using small gemfibrozil doses similarly as proposed previously for agents metabolised by other enzymes (Sellers *et al*., 2000). In addition, the formation of CYP2C8-mediated toxic metabolites of therapeutic drugs could possibly be reduced by gemfibrozil administration. E.g., neurotoxicity, which is currently the dose-limiting issue in the clinical use of the antineoplastic agent paclitaxel, has been shown to be associated with the *CYP2C8* genotype (Mielke *et al*., 2005; Gréen *et al*., 2008; Leskelä *et al*., 2011). If the CYP2C8-dependent paclitaxel metabolites are shown to be more neurotoxic than the CYP3A4-dependent metabolites, as currently thought, one therapeutic option in cancer patients receiving paclitaxel is to inhibit CYP2C8 by a small gemfibrozil dose thereby forcing paclitaxel to CYP3A4-mediated metabolism routes. This could be done by a separate formulation of gemfibrozil or a combined paclitaxel-gemfibrozil formulation. As new drugs are continuously recognised as being dependent on CYP2C8-mediated metabolism, there might be even more potential for the use of gemfibrozil as a CYP2C8 inhibitor in the future.

CONCLUSIONS

The following conclusions can be made based on the studies in this thesis:

1. Mechanism-based inhibition of CYP2C8 by gemfibrozil occurs rapidly in humans. The inhibitory effect developed to its maximum already in 1-3 h after gemfibrozil intake.

2. CYP2C8 inhibition after gemfibrozil treatment abates slowly. A full recovery of CYP2C8 activity, as measured by repaglinide metabolism, was achieved 96 h after cessation of gemfibrozil treatment. If CYP2C8-dependent substrate drugs, particularly those with a narrow therapeutic index (if the use with gemfibrozil is not contraindicated), are started during the first 1-3 days after stopping gemfibrozil treatment, the reduction of their doses should be considered.

3. Mechanism-based inhibition of CYP2C8 by gemfibrozil was shown to be dose-dependent. With increasing doses, both in single and multiple dose schemes, the extent of the inhibition increased. It was estimated that a level of ~50% inhibition of the CYP2C8 activity could already be obtained by a single 30 mg dose of gemfibrozil or by twice daily administration of << 30 mg of gemfibrozil. Further, > 90% of the CYP2C8 activity could be inhibited by a single dose of 900 mg or twice daily dosing of approximately 100 mg gemfibrozil.

4. The first-order degradation rate constant of CYP2C8 in humans is estimated at 0.00056 min^{-1} and the corresponding CYP2C8 enzyme turnover half-life at 22 h. These values can be used in the prediction of interactions caused by the mechanism-based inhibition of CYP2C8.

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